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Functional Motifs in SIAMESE, a Plant Cyclin-Dependent Kinase Inhibitor

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FUNCTIONAL MOTIFS IN SIAMESE, A PLANT CYCLIN-DEPENDENT KINASE INHIBITOR

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This work is dedicated to my parents for their immense support and love throughout my academic career

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ABBREVIATIONS

NASC	Nottingham Arabidopsis Stock Center
TAIR	The *Arabidopsis* information resource
SIM	Siamese
RBR	Retinoblastoma-related protein
APC/C	Anaphase promoting complex/Cyclosome
CDC	Cell division cycle
CCS52	Cell cycle switch protein52
CDKA;1	Cyclin dependent kinase A;1
CDKB	Cyclin dependent kinase B
CYC	Cyclin
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase enzyme
SMR	Siamese related protein
LGO	Loss of giant cells from organs
CAK	CDK activating kinase
SLC	Split luciferase complex
CKI	CDK inhibitor
ICK/KRP	Inhibitors/interactors of cyclin-dependent kinases/Kip-related protein

ABSTRACT

SIAMESE (SIM) and SIAMESE-RELATED-PROTEIN1 (SMR1), the founding members of the SIM/SMRs gene family, suppress mitosis and onset of endoreplication in the *Arabidopsis's* trichome and sepal development, respectively, and hence have been suggested to be CDK inhibitors. In this study, I have investigated the exact role of SIM and SMRs and their evolutionarily conserved function throughout land plant evolution. Using split luciferase complementation (SLC), I have shown that both SIM and a distantly related a bryophyte "*Physcomitrella patens"* SMR (pSMR1) interacts with multiple types of Cyclin Dependent Kinases (CDKs). I have multiple lines of evidence that establish SIM and SMRs as CDKs inhibitors and demonstrating that their evolutionary function is conserved.

Almost all SIAMESE-RELATED PROTEINS (SMRs) of *Arabidopsis* as well as a *SMR* from the bryophyte *Physcomitrella patens* complement the *sim* mutant phenotype strongly. Genetic studies of *sim* mutants in combination with *cyclind* and *cdkb1* mutants also support the conclusion that SIM inhibits the activity of both CDKA;1 and CDKB1;1-containing complexes. In an *in vitro* kinase assay, SIM inhibits CDK kinase activity; moreover, the *Physcomitrella* SMR also inhibits the same set of CYC/CDK complexes as SIM. These results indicate that SIM and other SMRs inhibit multiple CDK complexes and share a molecular mechanism that is conserved among all land plants. Finally, we have investigated the functional role of conserved protein sequence motifs in SIM. Two motifs, termed Motif-1 and Motif-2, play important roles in *SIM* function. Surprisingly, a motif previously thought to be a putative cyclin-binding motif is not essential for function of *SIM*. We have also identified a putative CDK phosphorylation site in Motif-1, and two nuclear localization sequences that are essential for *SIM* function.

The work described here gives new insights into the biochemical role of SIM in regulating the cell cycle. The conserved function of widely divergent *SMR*s indicates that this protein family plays important roles in all land plants. These studies will provide a foundation for future work on the biochemical functions of SIM in the cell cycle, as well as for understanding the roles of individual *SMR*s in plant growth and development.

CHAPTER 1
INTRODUCTION

1.1 Cell cycle, growth and development

Cell division, expansion, and differentiation are three essential processes for plant development. Through these processes, a single-celled zygote can achieve a well-organized multicellular form to allow the survival of an organism. How the cell cycle keeps pace with the development of an organ or an organism is a challenging question in developmental biology. Cell division and growth are tightly coupled processes and that can effect development directly or indirectly (Jakoby and Schnittger 2004; Kolly et al. 2005; Andriankaja et al. 2012). The importance of cell division is obvious in every living organism, and the number and orientation of cell division are essential for a plant form (Reddy et al. 2004; Petricka et al. 2009; Smolarkiewicz and Dhonukshe 2013). The importance of studying cell cycle regulation is diverse ranging from increasing crop yield, protection of plants from pathogen and biotic or abiotic stresses, to controlling cancer in humans. Unlike animals, plant development is postembryonic and there is a transition from embryonic stage to vegetative stage. Additionally, plants have unique features such as regeneration ability, sessile and autotrophic life style. The basic cell cycle regulatory mechanism is conserved in plants and animals; however, some significant differences exist.

1.2 Cell Cycle

The cell cycle is a fundamental process for the development of all organisms and consists of four phases: G1 (gap-1), Synthesis (S)-phase, G2 (gap-2) and Mitosis (M)-phase. G1 and G2 are gap or interphases where the cell prepares itself to enter into the S-phase or M-phase, respectively. S-phase is the phase in which DNA is synthesized, doubling the amount of DNA, and mitosis (M)-

phase is where chromosomes containing the duplicated DNA are separated and equally distributed between two daughter cells (Figure 1.1).

Transitions from G1 to S-phase and G2 to M-phase in the cell cycle are regulated by serine-threonine kinases known as Cyclin Dependent Kinases (CDKs) (Nigg 1995; Mironov et al. 1997; Morgan 1997). As the name indicates, the activity of these enzymes depends on the concentration of regulatory proteins known as cyclins. CYCD/CDKA complexes positively regulate the G1/S transition by phosphorylating RETINOBLASTOMA (RB) protein leading to dissociation of retinoblastoma from RB/E2F/DP complex (See Figure 1.1) (Boniotti and Gutierrez 2001), hence releasing the active E2F/DP transcription factor to activate the G1/S transition (Berckmans and De Veylder 2009). Unlike yeast, both animals and plants have the same RB/E2F/DP pathway to control the regulation of genes involved in G1/S transition. Plant homologs to pRB, which in animal cells is a tumor suppressor gene, are known as RETINOBLASTOMA-RELATED PROTEINs (RBRs). The *Arabidopsis* genome only encodes one RBR protein (Ebel et al. 2004).

The G2/M transition in plants is unique and appears to be controlled by increased transcription of CDKBs in place of the WEE1 kinase/CDC25 phosphatase pair that activates M-phase in animals and some fungi (Boudolf et al. 2006). CDKBs interact with CYCA and B to regulate the G2/M transition and M-phase progression, respectively (Boudolf et al. 2009; Xie et al. 2010; Vanneste et al. 2011). CYCA3 binds to CDKA;1 and may maintain S-phase progression (Schnittger et al. 2002; Dewitte et al. 2007; Boruc et al. 2010b; Boruc et al. 2010c; Van Leene et al. 2010).

The Anaphase Promoting Complex/Cyclosome (ACP/C) is an E3 ubiquitin ligase complex that plays a crucial role in mitosis and G1 by targeting specific cell cycle proteins for

proteolysis. The D-box, or "destruction box" that is found in most targets of APC has the amino acid sequence RxxLXXLXN, which is found near the N-terminus of cyclins CYCA and CYC B and in the other proteins recognized by the APC as substrate. Cell division cycle (Cdc20) and *CELL CYCLE SWITCH PROTEIN52* (known as Cdh1/FZR in animals) are two activators of APC and play the same role as their counterparts in animals. APC/C^{cdc20} targets are the mid-M phase proteins securins, cyclin A and B, and promote chromosome separation. APC/C^{ccs52} replaces APC^{Cdc20} in G1 and prevents the reaccumulation of mitotic cyclins (Tarayre et al. 2004; Fulop et al. 2005).

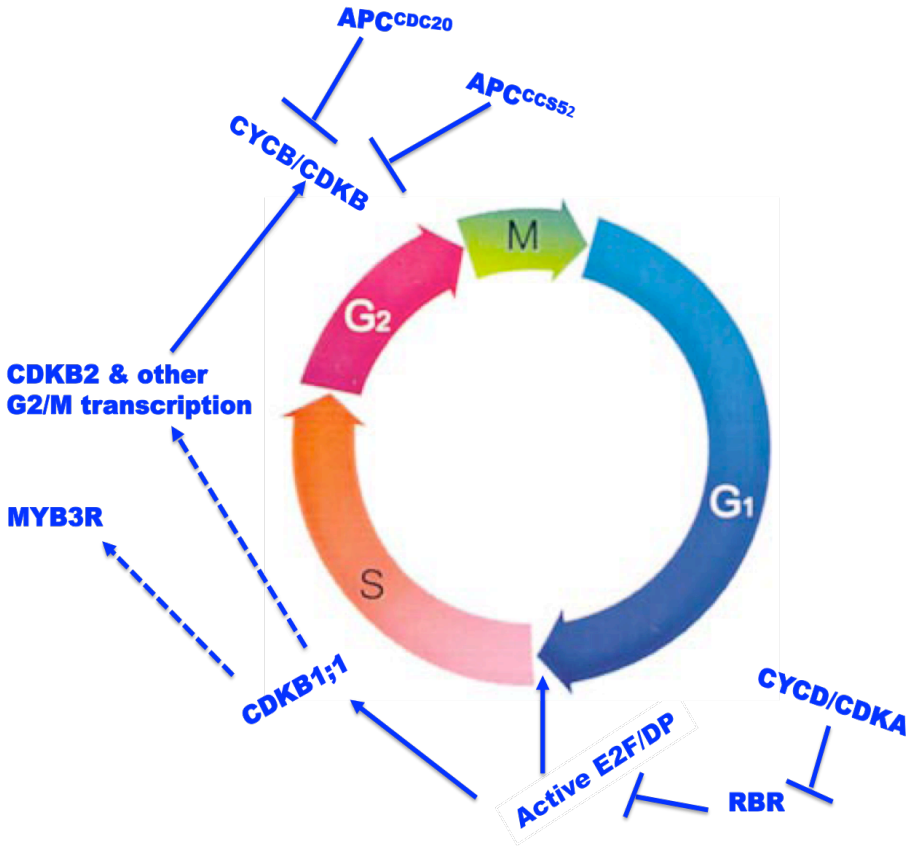


Figure.1.1 Simplified Plant Mitotic Cell Cycle, pointed and flat arrow indicates activation and inhibition, respectively.

1.3 Comparison of yeast, animal and plant cell cycles

Plants, yeast and metazoans share many aspects of the cell cycle, but there are a few significant differences among their cell cycle transition phases. Unlike yeast and animals, plants have a specific class of CDKBs that regulate the G2/M transition (Boudolf et al. 2004). Unlike metazoans, inhibitory phosphorylation of CDKA by WEE1 and activation by CDC25 is absent in plants (Boudolf et al. 2006). A unique class of CDKs inhibitors, the *SIAMESE/SIAMESE RELATED PROTEINS (SIM/SMRs)* gene family is only present in plants (Churchman et al. 2006). Geminin, an inhibitor of DNA replication, does not have a homolog in the plants. DNA damage response transcription factor class SUPPRESSOR OF GAMMA RESPONSE1 (SOG1) is a plant-specific regulator of the DNA damage checkpoints, whereas CHK1 and CHK2 kinases, which are signal transducers in the DNA damage response signaling pathway, the transcription factor P^{53} and the tumor suppressor P^{2} tumor suppressor are animal-specific (Yoshiyama et al. 2013).

1.4 Components of the plant cell cycle: cyclins

Arabidopsis provides the most thoroughly examined plant cyclin family. There are ten A-type cyclins (CYCAs) in *Arabidopsis* grouped in three different classes, based on sequence similarity, CYCA1, CYCA2 and CYCA3 (See table 1.1) (Chaubet-Gigot 2000; Vandepoele et al. 2002; De Veylder et al. 2007). B cyclins comprise three subclasses, CYCB1, CYCB2, and CYCB3. The majority of CYCAs and CYCBs contain a "destruction box" sequence (RxxLxxIxN or D-box) (Vandepoele et al. 2002). The Anaphase Promoting Complex (APC), which is an E3 ubiquitin ligase, targets the D-box sequences containing proteins for ubiquitination and degradation by the 26S proteosome (Yamano et al. 2004). With a few exceptions (for instance, CYCA3 expression peaks in S-phase), almost all CYCAs and CYCBs

have a similar cyclic expression pattern throughout the cell cycle, peaking in late G2 or M (See Figure 1.2) (Menges et al. 2005) and most CYCAs and CYCBs are thought to regulate entry into mitosis, with the exception of the CYCA3s, which may function to maintain DNA replication during S-phase (Yu et al. 2003; Takahashi et al. 2010).

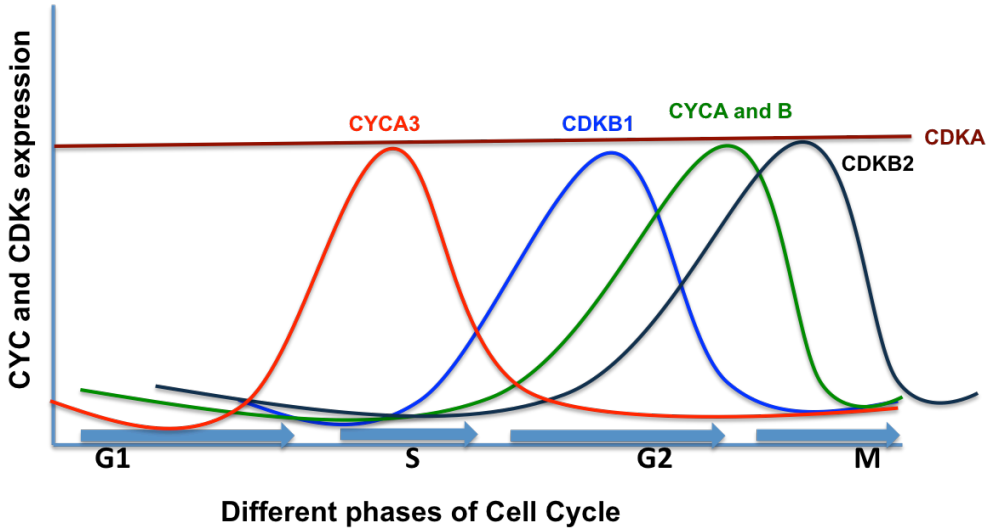


Figure 1.2 CYC and CDKs expression in typical cell cycle phases

The ten Cyclin Ds are classified into the seven groups: CYCD1, CYCD2, CYCD3, CYCD4, CYCD5, CYCD6, and CYCD7. CYCD3 and CYCD4 have three and two members respectively, while the other subgroups have only a single member (See table 1.1) (Vandepoele et al. 2002; Menges et al. 2007). Unlike most Cyclin As and Cyclin Bs, D-type cyclins lack a putative destruction box. Except *Arabidopsis* CYCD4;2, almost all CYCDs have a hydrophilic PEST region consisting of Pro (P), Gln (E), Ser (S) and Thr (T) amino acids (Menges et al. 2006; Menges et al. 2007). In addition, most CYCDs have the sequence LXCXE, which is a binding site for retinoblastoma like protein (Oakenfull et al. 2002). Plant D-type cyclins were identified by their ability to complement G1 cyclin mutants of yeast (Dahl et al. 1995; Soni et al. 1995).

Table 1.1 Cell Cycle Genes

Cell Cycle Genes	Description	Number in *Arabidopsis*
CYCD 1-7	D-type cyclins are specific to both G1/S and G2/M, expressed in response of external stimuli such as sucrose or development clue	7
CYCA 1-3	CYCA1 and CYA2 interact with CDKBs and negatively regulate endoreplication, CYCA3 interacts with CDKA;1 and upregulated at G1/S transition phase	3
CYCB 1-3	CYCB transcriptionally controlled by M-specific activator promoter elements (MSA) and destroyed by APC (Anaphase Promoting Complexes) ubiquitin ligase	3
CDKA;1	CDKA;1 interacts with D-type cyclins, phosphorylates and inactivates RBR	1
CDKB1	Expressed during S/G2 phase, two sub-classes CDKB1;1 and CDKB2;1	2
CDKB2	Expressed during G2/M phases, CDKB2;1 and CDKB2;1	2
RBR	RBR Binds to E2F and acts as transcriptional suppressor for wide range of genes, phosphorylated and activated by CYCD/CDKA;1 Complex	1
DP	Dimerization partners of E2Fs	2
WEE1	Unlike in animals, involved in DNA damage response rather than plant mitotic cell cycle	1
CDC20	Activator of Anaphase Promoting Complex (APC) and degrades CYCB to promote exit from Mitosis	6
CCS52	CDH1 homologs in plant, destruct CYCB to maintain low CDK activity during G1 and positively regulate endocycle	3

Expression of many CYCDs responds to the availability of nutrients and hormones, making CYCD/CDKs complexes key regulators of the cell growth through their regulation of the G1/S transition (Planchais et al. 2004; Menges et al. 2006; Kwon and Wang 2011).

1.5 Plant cell cycle components: CDKs

Plant CDKs have been classified as CDKA, CDKB, CDKC, CDKD, and CDKE based on their sequence similarity, with CDKAs and CDKBs being the main cell cycle regulators (Joubes et al. 2000). To distinguish animal and plant CDKs, numbers have been used for animal CDKs whereas combination of letters and numbers have been used for plant CDKs (Joubes et al. 2000). Plant CDKAs possess the PSTAIRE sequence in their cyclin binding domain as their counterparts in mammals (CDK1/2/4/6) and yeast (CDC2/CDC28), and play important roles in the G1/S and G2/M transition of the cell cycle (Inze and De Veylder 2006). *Arabidopsis* CDKA;1 interacts with different type of cyclins: D, A2, A3, and B, suggesting that activation of CDKA;1 may be involved from G1/S to mid M-phases of the cell cycle (Boruc et al. 2010c; Van Leene et al. 2010).

The mitotic CDKBs are unique to plants (Boudolf et al. 2006) and do not complement *cdc2/cdc28* yeast mutants (Imajuku et al. 1992; Fobert et al. 1996). CDKBs do not have the PSTAIRE sequence in their cyclin binding-motifs; instead they have PPTALRE (CDKB1) or PPTTLRE (CDKB2) (Joubes et al. 2000; Porceddu et al. 2001). CDKB1 (CDKB1;1 and CDKB1;2;) expression continues to rise through S phase and peaks in G2 phase, while CDKB2 transcription level peaks slightly later in M phase as shown in figure 1.2 (Menges et al. 2005). CDKB1 binds with A2 and B2 type cyclins, whereas CDKB2 binds exclusively to B1 cyclin in late M phases (Van Leene et al. 2010). Use of dominant-negative form of CDKB1;1 (N161) resulted in functional loss of CDKB1;1 blocked asymmetric division in guard mother cells

(GMCs) (Xie et al. 2010). CDKB2 is required for cell cycle progression and for proper organization of meristem structure, and loss of CDKB2 function resulted in disorganization of shoot apical meristem (SAM) structure (Andersen et al. 2008). CDKD and CDKF, a class of CDKs, known as CDK activating kinases, phosphorylate a critical threonine (T160) in the CDK T-loop region to activate CDKs (Umeda et al. 1998; Vandepoele et al. 2002; Shimotohno et al. 2006). CDKD is functionally similar to animal CAKs, whereas CDKF is plant specific (Boudolf et al. 2006) and shows functional similarity to the yeast CAK activating kinase (CAKK) (Umeda et al. 2005). CDK activity is dependent on phosphorylation/de-phosphorylation and interaction with the regulatory CYC proteins. CDK activating kinase (CAK) activates CDK by phosphorylating the threonine (T-160) located in T-loop (Shimotohno et al. 2006) and activity is inhibited by phosphorylation at the N-terminal Tyr residue.

1.6 Regulation of cell cycle

Cyclin concentrations fluctuate cyclically throughout the cell cycle, whereas CDKA;1 expression is constitutive throughout the cell cycle. B-type CDK expression peaks at specific (G2 and G2 to M phase, figure 1.2) cell cycle phases (Segers et al. 1996; Magyar et al. 1997; Umeda et al. 1999; Andersen et al. 2008). The activity of the CDKs decreases and increases in accordance with cyclin concentrations, and depends on the presence of CDK inhibitors and CDK activating Kinase (CAK) (Umeda et al. 2000; Vandepoele et al. 2002; Menges et al. 2005; De Veylder et al. 2011). Different combinations of cyclins (CYCs) and cyclin-dependent kinases (CDKs) regulate different stages and check points of the cell cycle (Inze and De Veylder 2006; Menges et al. 2006; Gutierrez 2009; Coudreuse and Nurse 2010). The maximum time of accumulation of the various cyclins specifically corresponds to their time of function in the cell cycle (Renaudin et al. 1996; Wang et al. 2004; Menges et al. 2005). A-type cyclins (CYCAs)

maintain DNA replication and promote mitosis, B-type cyclins (CYCBs) are thought to function primarily in mitosis, and D-type cyclins (CYCDs) are generally assumed to mainly function to regulate entry into S phase (Ito et al. 1997; Inze and De Veylder 2006; Dewitte et al. 2007; Scofield et al. 2013). The CYCD3;3/CDKA;1 complex can phosphorylate RBR protein and acts as a rate limiting regulator of the G1/S transition in Tobacco (Nakagami et al. 2002). CYCD plays a role in cell division during seed germination in Arabidopsis, and CYCD2;1 in combination with a KRP-type CDK inhibitor plays a key role in lateral root formation (Masubelele et al. 2005; Sanz et al. 2011). Overexpression of CYCD3;1 drives tissue culture cells from G1 into S phase (Dewitte and Murray 2003; Menges et al. 2006). CYCD4;1 makes a active complex with CDKB2;1, so CYCD4 type cyclins may be involved in the cell division, whereas CYCD4;2 is reported to be lacking PEST sequence and RBR binding motif (Kono et al. 2003; Kono et al. 2006). CYCD6;1 binds with A and B1 type CDKs and is involved in the root cortex/endodermis asymmetric division under the control of the SHORT-ROOT and SCARECROW (SCR) transcription factors (Sozzani et al. 2010; Cruz-Ramirez et al. 2012)

Genes specifically transcribed in G2-M phase (CYCB and CYCs, as well as the CDKBs) often have M-phase specific activator (MSA) elements in their promoter regions (Menges et al. 2005). These elements are the binding sites for MYB3R transcriptional factors (Ito 2005). Specific complexes, presumably CYC/CDK complexes, are thought to phosphorylate MYB3R proteins, activating them to transcribe CYCAs, CYCBs, CDKBs and other G2-M phase specific genes (Araki et al. 2004; Dissmeyer et al. 2007; Berckmans and De Veylder 2009).

Transition from one cell cycle stage to the next is unidirectional and irreversible. This irreversibility is achieved by the proteolytic destruction of regulatory proteins. The ubiquitin-proteasome pathway has emerged as a widespread proteolysis pathway. Ubiquitin, a highly

conserved 76 amino acid polypeptide in eukaryotes that tags a protein to be degraded in an ATP-dependent cascade of reactions includes three enzymes E1, E2 and E3. E3 is the most diversified enzyme in the ubiquitination cascade; more than one thousand different E3 ubiquitin ligases have been predicted in *Arabidopsis* (Mazzucotelli et al. 2006). Skip-Cullin-F-Box (SCF) and Anaphase Promoting Complex/Cyclosome (APC/C), and Cullin-RING ubiquitin ligases (CRLs) are some of the important E3 ubiquitin ligases that function in eukaryotic cell cycle. They use quite different mechanisms of substrate recognition and regulation of enzyme activity. Skp-Cullin-F-Box (SCF)-type is an ubiquitin protein ligase thought to target CDK inhibitors at the G1/S transition (Vodermaier 2004; Nakayama and Nakayama 2006)). Cdc20 and Cdh1/FZR (known as CCS52A in plants) are two important activators of the Anaphase-Promoting Complex/Cyclosome (APC/C) (Visintin et al. 1997), and degrade mitotic proteins such as securin and cyclins (Morgan 1999). CYCB destruction takes place in two phases: in the first phase co-activator Cdc20 catalyzes CYCB destruction that occurs at the mitotic spindle to promote exit from mitosis, while co-activator Cdh1 (CCS52A) catalyzes the second phase of CYCB destruction to maintain low CDK activity during G1 (Raff et al. 2002). Cdc20-APC/C complexes target proteins for destruction early, whereas Cdh1-APC/C complexes target proteins for destruction later in the exit from mitosis and in G1 (Sigrist and Lehner 1997; Vodermaier 2004; Nakayama and Nakayama 2006). In addition to the APC and SCF E3 ligases, CULLLIN RING ubiquitin ligases (CRLs) are another important class in plants and share a RING finger protein called RING box protein1 (RBX1) (Lechner et al. 2002). CULLIN4-RING FINGER-LIGASE regulates endoreplication in *Arabidopsis* trichome development, so CRL ligases may play role in endoreplication progression by inhibiting CDK activity (Roodbarkelari et al. 2010)

Cell cycle exit is not only controlled or regulated by one set of genes or extra-cellular or intra-cellular factors depending on the nature of cell, there might be several signals causing cell cycle exit such as reactive oxygen species (ROS) transcriptional repression of peroxidases, stress signaling, or CDK kinase inhibitors. *SMR5* and *SMR7* members of the *SIAMESE/SIAMESE RELATED PROTEINS (SIM/SMRs)* family of CDK inhibitors arrest the cell cycle proliferation in response to ROS induced by DNA damage or chloroplast dysfunction (Hudik et al. 2014; Yi et al. 2014). Recently, the involvement of other SMRs in cell cycle exit in response to ROS has been reported; for example SIM, the founding member of *SIM/SMRs* family, is directly controlled by the transcription factor UPBEAT1 (UPB1) that maintains ROS homeostasis in *Arabidopsis* roots (Hudik et al. 2014; Yi et al. 2014; Polyn et al. 2015). Cell cycle entry, maintenance and exit is coordinated and operated by many genes. The genes involved in the cell cycle exit are most interesting and would give new insight to understand the mechanism of cell cycle exit and entry into a differentiation phase of *Arabidopsis's* trichome development.

1.7 A cell cycle variant-endoreplication

Endoreplication is a cell cycle variant in which DNA is replicated without subsequent mitosis and cytokinesis, consequently doubling the DNA amount in each round of the cell cycle, and increasing cell size (Edgar and Orr-Weaver 2001; De Veylder et al. 2011; Fox and Duronio 2013). Three forms of endoreplication exist in organisms. The first is an endocycle, in which the cell does not enter into mitosis at all, so only S-phase and G-phases occur. The second form is endomitosis where condensation of chromosomes begins, but no separation to the daughter cells occurs because of early exit of M-phase (Nagata et al. 1997; Vitrat et al. 1998). The third form is re-replication, an aberrant re-initiation of DNA replication, where origins of replication are initiated more than once within single S-phase and the DNA content of a cell increases without

the recognizable genome doubling as seen in endocycle (Blow and Hodgson 2002; Zhong et al. 2003). Because of their similar biological out-come, all three phenomena are commonly known as endoreplication.

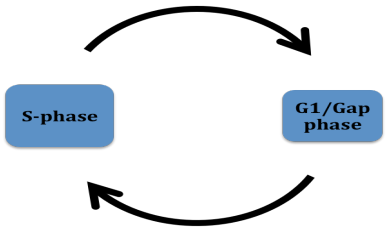


Figure1.3 Endoreplication: G2 and M phases are skipped**.**

Endoreplication is present in a wide range of living organisms from unicellular (such as ciliated protozoa and even in bacteria) to multicellular organisms; it is particularly frequent in plants (Mendell et al. 2008; Yin et al. 2010).

Endoreplication is a determinant in the process of differentiation and also in cell fate maintenance (Castellano Mdel et al. 2004; Lee et al. 2009b; Bramsiepe et al. 2010). Endoreplication occurs in many early developmental processes of plants and animals such as in endosperm development, hypocotyl growth, trophoblast differentiation during human embryo implantation, and in Drosophila follicle cells (Sarto et al. 1982; Kowles and Phillips 1985; Van't Hof 1999; Lilly and Duronio 2005; Lee et al. 2009a). Quantitative evidence has shown that DNA endoreplication leads to increase in neuronal size, and it results in body growth in adult mollusks (Yamagishi et al. 2011; Yamagishi et al. 2012)**.** In plants, endoreplication is common in tissues that develop mass very quickly or have a higher metabolic rate (Inze and De Veylder 2006). The volume to size ratio indicates that endoreplication might be necessary for the growth of large cells in plants (Kondorosi et al. 2000). Inhibition of endoreplication in the Drosophila salivary

gland, using DNA replication inhibitors, shows the requirement of high ploidy for both cell and organism growth (Edgar and Orr-Weaver 2001). Endoreplication may also support enhanced metabolic demand in a diverse set of symbiotic and parasitic interactions (Wildermuth 2010). In the nitrogen-fixing root nodules of *Medicago truncatula,* endoreplication is proportionally related to the nodule cell size and the efficiency of nitrogen fixation*,* and decreased endoreplication of root nodules leads to reduced cell size and nitrogen fixation efficiency (Kondorosi et al. 2000; Vinardell et al. 2003; Kondorosi and Kondorosi 2004; Yamagishi et al. 2012). Although most work suggesting a role for endoreplication in development is based on correlations, restoring endoreplication in *Arabidopsis* trichome mutants by destruction of mitotic cyclins can restore trichome cell fate, showing the requirement of endoreplication in cellular development (Bramsiepe et al. 2010).

1.8 CYC/CDK complexes in endoreplication

The mitotic cycle and the endo-cycle are thought to use the same machinery to control entry into S-phase. Available evidence indicates that suppression of CDK activity specifically at the G2-M-phase transition is required to switch mitotic cell division to endoreplication; therefore, endoreplication requires suppression of G2/M CDK activity either by degradation of mitotic cyclins, by expression of a CDK kinase inhibitor or both (De Veylder et al. 2011; Edgar et al. 2014).

A variety of evidence establishes the role of CYCs and CDKs in endoreplication. CYCD3;1 and CDKA;1 have been reported as rate limiting for G1-S and form a functional complex and also act a negative regulators of endoreplication (Dewitte et al. 2003; Menges et al. 2006; Bramsiepe et al. 2010; De Veylder et al. 2011; Collins et al. 2012). Overexpression of CYCD3, the interacting partner of CDKA;1, reduces endoreplication, decreases the proportion of

G1 cells and results in uncoupling of the growth and development in the *Arabidopsis* shoot apex (Dewitte et al. 2003). Additionally, exit of cell cycle and entry into differentiation stage is also highly compromised in the CYCD3;1 overexpressed leaves and cotyledon of *Arabidopsis* plants (Dewitte et al. 2003). CYCD3;1 is not expressed in wild type trichomes, but ectopic expression of CYCD3;1 changes unicellular endoreplicated wild type-trichomes to multicellular trichomes (Schnittger et al. 2002; Dewitte et al. 2003). A *cycd3;1-3* triple mutant increased endoreplication in *Arabidopis* petals and leaves (Dewitte et al. 2007). Moreover, it has been shown that the *cdka;1 Arabidopis* mutant has decreased ploidy level (Nowack et al. 2012)**.** The results indicate that CYCD3/CDKA;1 complexes are antagonists of the endocycle.

CYCA2;3 and CDKB1;1 also form a functional complex and are negative regulators of endoreplication onset (Boudolf et al. 2004; Boudolf et al. 2006). Reduction in CDKB1 activity causes arrest of plant cells in G2 (Porceddu et al. 2001; Boudolf et al. 2004). A dominant negative mutant of CDKB1 has increased ploidy level in *Arabidopsi*s leaves (Boudolf et al. 2004; Nowack et al. 2012). Moreover, co-expression of CYCA2;3 and CDKB1;1 increases ectopic cell division and reduces endoreplication in cotyledons, leaves and roots of *Arabidopsis* plants. Loss of CYCA2;3 function increases ploidy level, whereas overexpression of CYCA2;3 inhibits endoreplication in *Arabidopsis* leaves (Yu et al. 2003; Imai et al. 2006). Ectopic expression of mitotic cyclin CYCB1;2 without the destruction box switches endoreplication to mitotic cell division in *Arabidopsis* developing trichome (Schnittger et al. 2002; Kasili et al. 2010). Down-regulation of RBR stimulates endoreplication in maize endosperm, but it is not clear how it is related with CDKs (CDKA;1 or CDKBs) to inhibit the endoreplication in maize endosperm (Sabelli et al. 2013).

1.9 CDK inhibitors in endoreplication

CDK inhibitors play an important role in the onset of endoreplication. On the basis of interacting and inhibition activities, animal CDK inhibitors are divided in two classes: inhibitors of cyclin-dependent kinase4 **(**INK4), and CDK interacting protein/Kinase inhibitory protein (Cip/Kip) (Sherr and Roberts 1999). INK4 specifically inhibits CDK4 and CDK6 activity, whereas Cip/Kip family inhibits kinase activity of a wide range of mammalian CDKs (Lee et al. 1995; Carnero and Hannon 1998; Canepa et al. 2007).

In plants, the first CDK inhibitor family, INHIBITOR/INTERACTOR OF CYCLIN-DEPENDENT KINASES/KINASE-RELATED PROTEINS (*ICK/KRPs)*, was identified via their interaction with CDKA;1 in yeast two-hybrid screening (Wang et al. 1997; Lui et al. 2000; De Veylder et al. 2001). On the basis of exon-intron organization and the presence of conserved motifs, *Arabidopsis* has seven members of this CDK inhibitor family: *ICK1/KRP1, ICK2/KRP2, KRP3, KRP4, KRP5*, *KRP6*, and *KRP7* (De Veylder et al. 2001; Wang et al. 2008). In the plant development process, CKI/KRP (CDK kinase inhibitors/Kip Related Protein) affects the developmental process in a dose dependent manner and changes proliferating cell into differentiating cell (Weinl et al. 2005). There is strong evidence that KRPs increase endoreplication by inhibiting both the CYC/CDKA and CYC/CDKB kinase activities (Wang et al. 1997; Lui et al. 2000; Schnittger et al. 2003; Verkest et al. 2005a; Nakai et al. 2006). All KRPs have overlapping expression, with a few exceptions such as KRP 4 and 5 that express predominantly in dividing cells, KRP1 and 2 in differentiating cells, and KRP3, 6 and 7 that were observed in both dividing and differentiating cells (Ormenese et al. 2004). Mild over-expression of KRP1 and 2 inhibits mitotic dividing cells and promotes their entry into endocycle by inhibiting CYC/CDK complex activity, whereas strong overexpression of KRP1 or KRP2

completely inhibits the M and S-phase CDK activity, which leads to cell cycle arrest and produces aborted trichomes on *Arabidopsis* leaves (Lui et al. 2000; De Veylder et al. 2001; Schnittger et al. 2003; Verkest et al. 2005b; Weinl et al. 2005). Rice KRP1 regulates seed development (Barroco et al. 2006) and the interaction of KRPs with CYCDs confirms their regulatory role in cell cycle entry (Boruc et al. 2010a; Van Leene et al. 2010). KRP2 interacts with CYCD2;1 and suppresses lateral root primordial division formation by restricting S-phase entry (Sanz et al. 2011).

Little is known regarding the regulation and stability of *ICK/KRP*, although MG132, an inhibitor of the 26S proteosome, increases the stability of both ICK1/KRP1 and ICK2KRP2 in seedlings (Verkest et al. 2005a). In addition, KRP6 and KRP7 are the main targets of SCF^{FBL17} ubiquitin E3 ligase in *Arabidopsis* pollen (Kim et al. 2008; Gusti et al. 2009). Skp-Cullin-F-Box (SCF) proteins are a class of ubiquitin protein ligases that also play an important role in cell cycle progression (Nakayama and Nakayama 2005).

Another class of putative CDK kinase inhibitors in plant is the SIM/SMRs family. The *SIAMESE* gene was identified as an *Arabidopsis* trichome mutant suggesting a role for SIAMESE as negative cell cycle regulator, which in turn promotes endoreplication. The exact role of SIAMESE is under investigation; however, the *sim* mutant trichome phenotype and alignment of SIM and SIAMESE RELATED PROTEINS suggest that SMR family members are CDK inhibitors.

1.10 The *Arabidopsis* trichome as a model

Trichome is derived from the Greek word, *trichos,* for hair. Trichomes are the hairs present on plant leaves and stem. Taxonomically, trichomes are very important because

approximately 300 types of trichomes are described in the botanical literature to identify variations in trichomes.

Functionally, trichomes protect plants from heat, sunlight, herbivores, and increase tolerance to freezing conditions (Quinn et al. 2010; Runyon et al. 2010; Yan et al. 2012; Kazemi-Dinan et al. 2014). Glandular, non-glandular (most Cucurbita plants), single celled (*Arabidopsis*) and multicellular trichomes (Tobacco and tomato) are present among plants and almost every terrestrial plant has trichomes. Like *Arabidopsis* trichomes, cotton *(Gossypium hirsutum)* seed also has single celled trichomes. *Arabidopsis* trichomes are present on the cauline and rosette leaves, sepal and the stem, but there are no trichomes on the cotyledons and hypocotyls. Arabidopsis trichomes are stiff to touch and on the leaf or stem can be easily seen with naked eyes. Trichomes are easily observable under light/dissecting microscopes, and are dispensable under laboratory conditions. In the lab, for the experimental purpose, they are ideal because making mutants that affect trichomes does not affect other aspects of plant development (Marks 1997).

The trichome is a special cell on the *Arabidopsis* leaf epidermis (Figure 1.4A), which stops mitotic division, but DNA keeps replicating by endoreplication. A protodermal cell becomes larger with every round of DNA duplication and as a result a three branched aerial trichome originates perpendicular from a uniform field of epidermal pavement cells (Hulskamp et al. 1994)). Because of endoreplication, DNA amounts in a trichome reach up to 32C and sometimes 64C (1C is the amount of haploid genome).

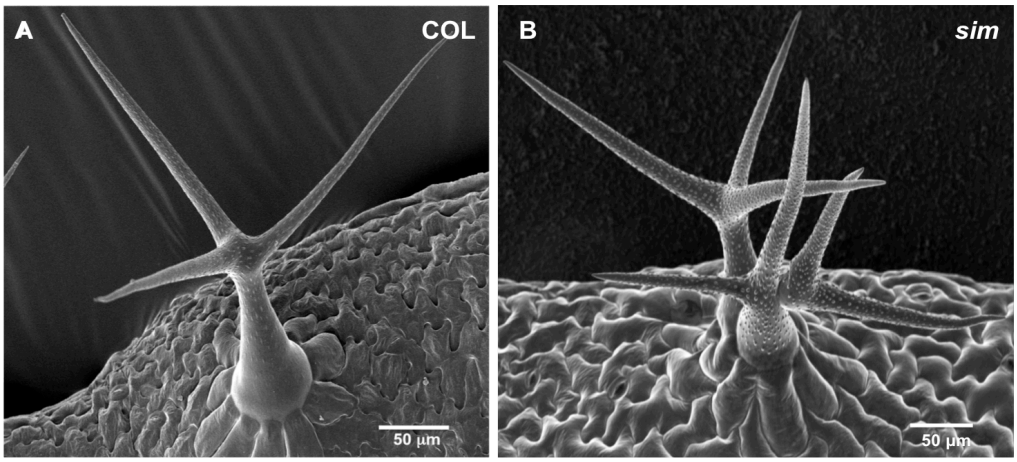


Figure 1.4 SEM images of Trichomes (A) Col, and (B) *sim* trichome (observed and identified by Walker et al. 2000; Churchman et al. 2006). Scale Bar is 50µm for both.

The *Arabidopsis* trichome is a large unicellular, polyploid cell that serves as an ideal model to study cell expansion and differentiation, including all aspects of cell development, cell polarity and cell cycle at single cell level (Schellmann and Hulskamp 2005; Pesch and Hulskamp 2009; Szymanski and Cosgrove 2009). Trichomes are well spaced at a regular distance on the epidermis of the leaf and never make a cluster or occur next to each other (Larkin et al. 1996; Hulskamp 2004). The genes involved in trichome development are well-studied and categorized into three classes on the basis of the different developmental stages. First, the patterning genes, which are responsible for the patterning and spacing of the trichome initiation sites, second are the branching genes and the third group of genes regulate endoreplication; all these genes are interconnected one way or another in the development of a trichome (Schnittger et al. 1999; Pattanaik et al. 2014).

1.11 Developmental genes and endoreplication in trichome development

Exiting mitosis and starting endoreplication is a unique phenomenon responsible for the differentiation of a cell. Endoreplication significance is well documented and widely reported among organisms, but the mechanism needs more investigation. Several genetic pathways along

with other factors are controlling endoreplication in the *Arabidopsis* trichomes. Available evidence indicates that trichome patterning genes are also involved in the endoreplication. Mutations in the *GLABRA3 (GL3)* trichome patterning gene, a positive regulator of trichome development, reduce ploidy level, whereas mutations in *TRIPTYCHON (TRY),* another patterning gene, lead to an increase in endoreplication (Hulskamp et al. 1994; Szymanski and Marks 1998; Perazza et al. 1999; Schnittger et al. 1999). *GLABRA1 (GL1)* and *TRANSPARENT TESTA-GLABRA (TTG)* interact with each other and are positive regulators of endoreplication (Schnittger et al. 1999). The *KAKTUS (KAK)* gene encodes putative E3 ligase, which regulates the progression of endoreplication by ubiquitination and subsequently degradation of the proteins involved in endoreplication; *Kak* trichomes contain twice the DNA of wild trichome cells (Downes et al. 2003; El Refy et al. 2003). *SPINDLY (SPY)* is a negative regulator of gibberellin (GA) signaling and encodes a putative O-linked N-acetyl-glucosamine (GlcNAc) transferase (Perazza et al. 1999; Shimada et al. 2006). The SPY gene is a repressor of gibberellic acid (GA) signaling, therefore mutated *spy* resulted in increased endoreplication in trichomes and the plant phenotype looks similar to that caused by a high concentration GA treated wild type plant (Jacobsen and Olszewski 1993). The *BRANCHLESS TRICHOMES (BLT)* gene seems to co-ordinate between endoreplication and branching in trichome development (Kasili *et al.,* 2011).

Many cell cycle genes and gene regulating endoreplication process have been reported to be involved in trichome development. Loss-of-function mutations in the *SIM* gene, which encodes a putative CDK inhibitor protein, change unicellular trichomes to multicellular trichomes by triggering mitosis (Churchman et al. 2006). Overexpression CYCD3;1 also gives same phenotype as the *sim* mutant, as does overexpression of a truncated CYCB1;2, without its D-box, under the trichome-specific *GL2* promoter.

1.12 SIM and SMR gene family

A recently discovered negative cell cycle regulator, SIAMESE is important in switching normal mitotic division to endoreplication. The *SIM* gene was isolated in the Larkin lab and identified as a regulator of endoreplication in the developing trichomes of *Arabidopsis thaliana*. *SIM* is functionally conserved in rice, based on the observation that the rice *SIM* homolog EL2 can complement the *Arabidopsis sim* mutant (Peres et al. 2007). SIM protein interacts with cyclin/CDK complexes (Churchman et al. 2006; Van Leene et al. 2011), and presumably works as a CDK inhibitor to cause onset of endoreplication in the developing *Arabidopsis* trichome.

The predicted SIM protein has five short sequence motifs that are conserved with other members of the SMR family. It also has limited similarity with another plant CDK inhibitor family, the KRPs. Motif-4 of SIM **(**EIFRFFSSVY**)** shows similarity to a motif found in the KRP family. Motif-1 and Motif-2 might be involved in the protein regulation and are most conserved, while the roles of motif-3 and motif-5 were unknown at the start of this work.

SIAMESE related proteins (SMRs) are members of the SIM/SMRs family and their function is also not well documented yet, but on the basis of this study, we have enough evidence to establish SIM as a CDK inhibitor and to show that all SIM/SMRs family members work by a molecular mechanism that is conserved in all land plants. Using site directed mutagenesis and phosphomimic approach, I have identified that threonine-35 phosphorylation, in Motif-1, is essential for the function of the SIM and may be a potential CDK phosphorylation site. With the help of systematic deletion and site directed mutagenesis scanning of the SIM protein, I have established that Motif-1 and 2 are essential for SIM function, and that SIM has two nuclear localization sequences in the Motif-3 and 5.

CHAPTER 2
MATERIAL AND METHODS

2.1 Plant materials and growth conditions

The Col-0 ecotype was used as the wild-type control for all experiments. The *sim-1* allele has been previously described (Churchman et al. 2006). The *cdka;1-1* (SALK_ 106809), *cdkb1;1-1* (SALK_073457) *cdkb1;2-1* (SALK_ 133560), *cycd3;1-1* (SET4061), *cycd3;2-1* (5580) and *cycd3;3-1* (N174667) have all been described previously (Nowack et al. 2006; Dewitte et al. 2007; Nowack et al. 2012). The cyclin D3 triple mutant *cycd3;1-3* was a kind gift from Drs. Walter Dewitte and James Murray (University of Cambridge, UK). The T-DNA insertions in *smr1* (SALK_033905) and *smr2* (SALK_124828C, SALK_006098C) were obtained from the Nottingham Arabidopsis Stock Center (NASC). All T-DNA and Ds insertion genotypes were confirmed by PCR using the primers specific for the wild-type allele and for the T-DNA allele, using primers described in Table 2.1. The *sim-1* allele was also genotyped via PCR. The *sim-1* allele changes the start codon to ATA (Churchman et al. 2006), creating a new BglII restriction site. Unfortunately, another BglII restriction site is located only 22 bp away from this new site. Therefore, primers were designed such that a 180 bp PCR fragment was produced from both wild-type and mutant DNA, and the pre-existing BglII restriction site was destroyed by a mismatch in one of the primers (Table 2.1). Digestion of the PCR product from the mutant allele with BglII results in a two fragments of 144 bp and 36 bp, while the PCR product of the wild-type allele is not cleaved by the enzyme.

For complementation experiments, crosses and most other experiments, plants were grown on soil as previously described (Larkin et al. 1999). For kinematic analysis of leaf growth, plants were grown for 26 days as follows: seeds were sterilized in 70% ETOH for 1 min and in

50% bleach for 10 min then rinsed 4x with dH_2O. The seeds were sown on half-strength Muraschige and Skoog (MS) germination medium (Muraschige & Skoog 1962) supplemented with 1% sucrose and 0.8% plant tissue culture agar. After 44h of stratification (DAS) in the refrigerator, the plates were placed horizontally on cooled benches in a growth chamber kept at 22^0 C under long-day conditions (16 h of light/8 h of darkness, 80-90 $\mu mols^{-1}m^{-2}s^{-1}$ photosynthetically active radiation, supplied by fluorescent tubes (Osram Lumilux, Cool white).

2.2 Generation of transgenic lines

Coding regions of *SIAMESE-RELATED PROTEINS (SMRs)* were synthesized by Integrated DNA Technologies and PCR amplified with appropriate primers (Table 2.1), and inserted into the vector (pENTR™/D-TOPO®) using a pENTR™ Directional TOPO® Cloning Kit (Life Technologies). Error-free entry clones were confirmed by sequence analysis. An LR clonase reaction was performed to insert genes into the GATEWAY™ binary T-DNA destination vector pAMPAT-PRO_{GL2}, which contains the *GL2* promoter (Weinl et al. 2005). The resulting *SMR*-expression constructs were introduced into *Agrobacterium tumefecians* strain *GV3101 pMP90RK* by electroporation (Weigel and Glazebrook 2006), and subsequently used to transform *sim-1* homozygous mutant *Arabidopsis* plants via the floral dip method (Clough and Bent 1998). Seeds were planted on soil and transgenic plants were selected with 1mM BASTA™ spray. Complementation of the *sim* trichome phenotype was initially scored in T1 generation transgenic plants, and 12 to 18 primary transformants per construct were screened for segregation of a single BASTA™-resistant insert in the T2 generation, and the three most strongly complementing single insert lines were used to produce homozygous T3 lines. In all complementation experiments, the plants were confirmed to be *sim-1* homozygotes as described above.

2.3 Scanning Electron Microscopy (SEM)

For SEM, the first leaves of two week-old Arabidopsis plants were mounted on the specimen stubs using double-stick tape, and observed under high vacuum mode at 5.0 kV in a JEOL JSM 6610LV Scanning Electron Microscope, working quickly to avoid drying and damage from the beam.

2.4 Split-Luciferase Assay

The dual expression series vectors pDuEx-AN6, pDuEx-DN6, pDuEx-AC6, and pDuExDC6 (Fujikawa and Kato 2007), which carry the N-terminus (Amino-acids 1-229, Nluc) or C-terminus (Amino-acids 230-311, Cluc) of the *Renilla reniforms* luciferase coding region, respectively, were used for the Split Luciferase Complementation Assays. SIM and CDKs were introduced into their respective vectors by GATEWAY™ cloning (Life Technologies). Proper orientation and correct sequence of the inserts in all constructs was confirmed by sequence analysis. The assays were carried out in 96-well plates. Plasmids carrying coding regions of proteins to be tested were introduced into protoplasts using polyethylene glycol-mediated transfection and incubated overnight at room temperature (Fujikawa and Kato 2007). After 14 to 18 hours incubation, a coelenterazine derivative, ViviREN Live Cell substrate (Promega**)** was added to the protoplasts, and luminescence was detected with a VERITAS micro-plate luminometer as described previously (Fujikawa and Kato 2007).

2.5 Site Directed Mutagenesis

Site-directed mutants were created either using a QuickChange II™ site-directed mutagenesis kit (Agilent) or were ordered as complete synthetic coding regions (Integrated DNA Technologies). Error free entry clones were confirmed by sequence analysis prior to performing LR clonase reactions to insert genes into the GATEWAY™ binary T-DNA destination vector

pAMPAT-PRO$_{GL2}$, which contains the pGL2 promoter. The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain *GV3101 pMP90RK* by electroporation and used for transformation of *Arabidopsis* plants via the floral dip method (Clough and Bent 1998). Seeds were planted on soil and transformants were selected with 1mM BASTA^{TM}. Complementation of the *sim* trichome phenotype was initially scored in T1 generation transgenic plants, and 12 to 18 primary transformants per construct were screened for segregation of a single BASTA^{TM}-resistant insert in the T2 generation, and the three most strongly complementing single insert lines were used to produce homozygous T3 lines. In all complementation experiments, the plants were confirmed to be *sim-1* homozygotes by PCR genotyping.

2.6 Multisite Gateway Cloning

Enhanced Yellow Fluorescent Protein (eYFP) was fused to the N-terminus of wild-type or mutant *SIM* coding regions using a Multisite-Gateway^{TM} cloning kit (Invitrogen). The *eYFP* coding region was isolated from the vector pSAT6-EYFP-C1 and cloned in pENTR^{TM}/D-TOPO® (Life Technologies). The *eYFP* coding region and error-free pDONR221 *SIM* clones were confirmed by sequence analysis. To produce *attB* flanked PCR products, primers (Table 2.1) were designed according to MultiSite Gateway® manual guidelines. The aatB1-SIM-aatB5r and attb5-eYFP-attb2 PCR products were inserted in the pDONR221^{TM} P1-P5r and pDONR^{TM} 221 P5-P2 MultiSite Gateway® Pro Donor vectors, respectively. The *YFP*- and *SIM*-containing donor clones were then introduced into the pAMPAT-PRO$_{GL2}$ destination vector as described by the manufacturer (Life Technologies).

2.7 Light Microscopy

Light microscopy was performed with a Leica DM RXA2 light microscope equipped with differential interference contrast and epifluorescence optics, using 20x objective, and

images were captured using a SensiCam QE 12-bit, cooled CCD camera and analyzed with SlideBook software from 3I (Denver, CO). Nuclei per trichome initiation site were counted using first leaves stained with 4′, 6-diamidino-2-phenylindole (DAPI), as described previously (Walker et al. 2000).

2.8 Sequence collection, multiple sequence alignment and phylogenetic analysis

BLAST and PSI-BLAST searches were performed among all land plants in GeneBank (http://www.ncbi.nlm.nih.gov/) Refseq database, Phytozome (http://www.Phytozome.net), and PLAZA (http://bioinformatics.psb.ugent.be/plaza/) databases for putative SMRs using the known *A. thaliana* SMR genes. Then, using custom scripts, we filtered the dataset to give a unique non-redundant set. Based on their taxonomic diversity, 85 putative SMRs from seven plant genomes were selected for further analyses.

Multiple sequence alignment was performed initially with Clustal Omega (Sievers et al. 2011) and further improved using Rascal v1.34 (Thompson et al. 2003). A Neighbor-Joining tree was constructed using MEGA 6 (Tamura et al. 2013) with 85 unique sequences. The distance matrix from the pairwise sequence comparisons was used to create the distribution for the PCA analysis. The evolutionary distances were computed using p-distance and pairwise deletions. All ambiguous positions were removed for each sequence pair. Bootstrap analysis was performed with 1000 replicates.

2.9 CDK Kinase Assay

The SIM was subcloned, by Gateway®, into the pDONR221 vector (Life Technologies), and PpSMR12 was subcloned, into pENTR, by TOPO® Cloning, An entry clone, pDONR221-GFP (S65T), was obtained from Dr. Akira Iwase. Resulted clones were inserted in a destination

vector pHGGWA (Busso et al. 2005), by using LR Clonase II (Life Technologies). Error-free destination clones were confirmed by sequence analysis.

To express His:GST-fused proteins, *E. coli* SoluBL21 cells (AMS Biotechnology) were transformed with the resulting destination clone. *E. coli* cells were grown in LB medium containing 100 mg/l ampicillin at 37ºC until OD600=0.6 and the production of the fusion protein was induced by adding 0.3 mM IPTG overnight at 18ºC. Cells were harvested by centrifugation and re-suspended in Ni-NTA binding buffer (50 mM NaH_2PO_4, 100 mM NaCl, 10% (v/v) glycerol, 25 mM imidazole, pH 8.0) containing protease inhibitors (complete ETDA-free; Roche), and lysed by sonication (Digital Sonifier® 450D, BRANSON). After addition of Triton X-100 to 0.2% (w/v), the cell slurry was incubated at 4ºC and clarified by centrifugation. The supernatant was passed through a column packed with Ni-NTA Agarose resins (Qiagen), which was washed sequentially with Ni-NTA binding buffer, and eluted with Ni-NTA elution buffer (Ni-NTA binding buffer containing 200 mM imidazole) and the buffer was exchanged to kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA) with a PD-10 column (GE Healthcare). After each complex was concentrated with a VIVASPIN 2 (sartorius), the concentration of proteins was calculated by using BSA as a standard.

After CDK complexes were expressed and purified by using a system as described previously (Harashima and Schnittger 2012), ATP was added to 2 mM, and the complexes were incubated for 1 h at 30ºC. The reaction was then further purified with a column packed with Strep-Tactin sepharose resins (IBA), which had been equilibrated with kinase buffer. CDK complexes were eluted with kinase buffer containing 2.5 mM desthiobiotin. After measuring the concentration of the complexes with Bradford Reagent (Sigma) using BSA as a standard, the aliquoted complexes were frozen in the liquid nitrogen and stored at -80ºC until use.

The kinase assays were carried out with 15 nM of kinase, 2 µg of histone $H1^0$ (NEB) as a substrate, 92.5 kBq of [γ-^{32}P] ATP (PerkinElmer), and SIM or PpSMR12 proteins per reaction in a final volume of 20 µl. To assay for the inhibitory activity of SIM, purified HisGST-SIM, HisGST-PpSMR1, or His GST–GFP fusion proteins were added to the kinase reactions before the addition of the substrate.

After incubation for 30 min at 30ºC, kinase reactions were stopped by adding Laemmli sample buffer (Bio-Rad) and boiled. Samples were separated on 12% TGX gels (Bio-Rad), and after the gels were stained with Bio-Safe™ Coomassie G-250 Stain (Bio-rad), they were dried with Hydro-Tech™ Gel Drying System (Bio-Rad). Radioactive histone $H1^0$ proteins were detected using a Typhoon™ FLA-7000 system (GE Healthcare).

2.10 Transient expression in Nicotiana benthamiana

Tobacco (*Nicotiana benthamiana*) seeds grown on the soil in the growth chamber at 21-22^{o}C with 24 hours light. After 14 days plant were transferred in a single with the same soil. One to two months old plants were used for the infiltration. Solution was made, and Infiltration was done as described previously (Pusch et al. 2011).

Table 2.1 listed all the primers used in this study

Genotype primers

S. N	Genes Name	Forward Primer	Reverse Primer
1	AtCYCD3;1	5'-GCGATTCGGAAGGAGGAAGA-3'	5'-GCTGCGGCAACTACTGATGG-3'
2	Atcycd3;1	5'-TAAAAACCATCTCTCGGCCTTCTCTCAT-3'	5'-ACGGTCGGGAAACTAGCTCTAC-3'
3	AtCYCD3;2	5'-TGGCTTTGGAGAAAGAGGAA-3'	5'-GGGAAACCTCACCAGCAATA-3'

Continued Table 2.1 Genotype primers

SN	Gene name	Forward Primer	Reverse Primer
4	Atcycd3;2	5’-TCGGGCCTAACTTTTGGTG-3’	5’-TCACAGGGTGCATTCTCC-3’
5	AtCYCD3;3	5’-TCGTGAAAAGGCTCTTGATTGG-3’	5’-CGATCGGACTAGCGGGTTGT-3’
6	Atcycd3;3	5’-ACGGTCGGGAAACTAGCTCTAC-3’	5’-CGATCGGACTAGCGGGTTGT-3’
7	sim-1	5’-GACAGAAGCACATATA TATAAGAAACCC-3’	5’-GGAAATTCAAGATGGGCTGAT CTTGTATTAAATC-3’
8	CDKB1;1	5’-GCTTACCAATTGAGAACAACTGA TTC-3’	5’-TGT CTT TGA GCA GCC ATC TGT GTT G-3’
9	CDKB2;1	5’-TTT TTG TAC TCA GGG CCG GCT TTA C-3’	5’-GGTTCAAAACAAATTATCATCA ACTAGG-3’
10	Cdkb1;1	5’-GCGTGGACCGCTTGCTGCAACTC TCTCAGG-3’	5’-TGT CTT TGA GCA GCC ATC TGT GTT G-3’
11	Cdkb2;1	5’-GCGTGGACCGCTTGCTGCAACTC TCTCAGG-3’	5’-TTT TTG TAC TCA GGG CCG GCT TTA C-3’

Continued Table 2.1 SMRs primers

SN	Genes name	Forward prime	Revers primer
1	AtSMR1	5’-CACCATGGATCTTGAATTACTAC AAGA-3’	5’-TCATCTTCGAGAACAATAAGGGT AAC-3’
2	AtSMR2	5’-CACCATGTCTAAGCTTCTCGAGA C-3’	5’-TCAGGCACTATTACTCCTTCGTTT CTT-3’
3	AtSMR3	5’-CACCATGGCAGAGATCTGCTGCG T-3’	5’-TTATTGTTTCCGTGACTTCTTGAT CCTACG-3’
4	AtSMR4	5’-CACCATGGAGGTGGTGGA-3’	5’-CTAAGCGCAAGCTTCTCTTCGA-3’
5	AtSMR5	5’-CACCATGGAGGAGAAAAACTAC GACGAC-3’	5’-CTAGGTTGCCGCTTGGGAGGC-3’

Continued Table 2.1 SMRs primers

SN	Gene name	Forward primer	Reverse primer
6	"Spliced" AtSMR5	5'-CACCATGGAGGAGAAAAACTACGACGAC-3'	5'-TCACAATCTAAGCGCAACTAGGTTGCC-3'
7	AtSMR7	5'-CCACATGGGAATTTCGA-3'	5'-TTAACGGCGTTGTATAAACA-3'
8	AtSMR11	5'-CACCATGGAGCAAGAAGAACCATGTGAA-3'	5'-TCAGAAGAGTTTTCTTCTTAATCCAGAATC-3'
9	AtSMR13	5'-CACCATGGCATCAAAAGGAAAGAAAGAAAC-3'	5'-TTATTTATTATTTTGTTGACCAAGTGCGAAAACG-3'
10	Physcomitrella patents PpSMR	5'-CACCATGTCACCCACAACATCAGGTTG-3'	5'-TTAACTTCCCACTTGCACTTTGTGCTGG-3'

Continued Table 2.1 Site Directed Mutagenesis Primers

S.N	Genes Name	Forward Primer	Reverse Primer
1	SIM-T34A	5'-CGGCGGCTGCACCGCTCCCACTTCTTC-3'	5'-GAAGAAGTGGGAGCGGTGCAGCCGCCG-3'
2	SIM-T35D	5'-GCGGCGGCTGCACCGATCCCACTTCTT CCG-3'	5'-CCGAAGAAGTGGGATCGGTGCAGCCGCCGC-3'
3	SIM-T35E	5'-GGCGGCGGCTGCACCGAACCCACTTCTT CCGAC-3'	5'-GTCGGAAGAAGTGGGTTCGGTGCAGCCGC CGCC-3'
4	SIM-P51-54A	5'-CCACCACAGCCACCACTGCTGCTGCTGC AGCGCAGAAACCC-3'	5'-GGGTTTCTGCGCTGCAGCAGCAGCAGTGGTGGCTGTGGTGG-3'
5	SIM-C72AR74AL76A	5'-CGTCGTCTCTCGGCATCAGATCTGCCAA GGCAAAGGCTATGACGTCATTGTCCAAGTATG-3'	5'-CATACTTGGACAATGACGTCATAGCCTTTGCCTTGGCAGATCTGATGCCGAGAGACGACG-3'

Continued Table 2.1 Site Directed Mutagenesis Primers

SN	Gene name	Forward primer	Reverse primer
	SIM-K73AK75A	5'-GTCTCTCGGCATCAGATCTTGCGCG AGAGCGCTTATGACGTCATTGTCCAAG-3'	5'-CTTGGACAATGACGTCATAAGCGCTC TCGCGCAAGATCTGATGCCGAGAGAC-3'
7	SIM-R74AL76A	5'-CTCTCGGCATCAGATCTTGCAAGGCAAAG GCTATGACGTCATTGTCCAAGTATG-3'	5'-CATACTTGGACAATGACGTCATAGCC TTTGCCTTGCAAGATCTGATGCCGAGAG-3'
8	SIM-R74A	5'-CGGCATCAGATCTTGCAAGGCAAAGCTT ATGACGTCATTG-3'	5'-CAATGACGTCATAAGCTTTGCCTTGC AAGATCTGATGCCG-3'
9	SIM-L76A	5'-CATCAGATCTTGCAAGAGAAAGGCTATGACGT CATTGTCCAAGTAT-3'	5'-ATACTTGGACAATGACGTCATAGCCTTTC TCTTGCAAGATCTGATG-3'

Continued Table 2.1 Site Directed Mutagenesis Primers

SN	Genes name	Forward Primer	Reverse Primer
10	SIM-96A	AGATGAGATCGAGCGGTTCGCCTCCTCT GTTTACAACCAA-3'	5'-TTGGTTGTAAACAGAGGAGGCGAAC CGCTCGATCTCATCT-3'
11	SIM-95A	5'-CAAAGATGAGATCGAGCGGGCCTTCTCC TCTGTTTACAAC-3'	5'-GTTGTAAACAGAGGAGAAGGCCCGC TCGATCTCATCTTTG-3'
12	SIM-F95-2	5'-TCAACAAAGATGAGATCGAGCGGGCCGCCTCC TCTGTTTACAACCAAACGA-3'	5'-TCGTTTGGTTGTAAACAGAGGAGGCGGCC CGCTCGATCTCATCTTTGTTGA-3'
13	SIM-E91AE93A	5'-CATCGTCAACAAAGATGCGATCGCGCGGTTCT TCTCCTCTG-3'	5'-CAGAGGAGAAGAACCGCGCGATCGC ATCTTTGTTGACGATG-3'
14	SIM-C72A	5'-GTCTCTCGGCATCAGATCTGCCAAGAGA AAGCTTATGACG-3'	5'-CGTCATAAGCTTTCTCTTGGCAGATCT GATGCCGAGAGAC-3'

Continued Table 2.1 Site Directed Mutagenesis Primers

SN	Gene name	Forward primer	Reverse primer
15	SIM-T34A	5'-GGCGGCGGCTGCGCCGCTGCCACTTCT TCCG-3'	5'-CGGAAGAAGTGGCAGCGGCGCAGC CGCCGCC-3'
16	SIM-120A	5'-TGGCCAAGCGGCGAAGAGCTTTCCGTTC TTGTTCAC-3'	5'-GTGAACAAGAACGGAAAGCTCTTCGC CGCTTGGCCA-3'

CHAPTER 3
FUNCTIONAL CONSERVATION IN THE *SIAMESE-RELATED* FAMILY OF PLANT CYCLIN-DEPENDENT KINASE INHIBITORS

3.1 Introduction

The proper regulation and maintenance of the cell cycle is crucial throughout the life cycle for the survival of multicellular organisms. Progression through different cell cycle checkpoints is controlled by serine/threonine kinases known as Cyclin-Dependent Kinases (CDKs). CDK activity is regulated at the posttranslational level by binding regulatory subunits called cyclins (CYCs), by phosphorylation by CDK-activating kinases (CAKs), and by CDK inhibitors (Pines 1995; Pavletich 1999; Vandepoele et al. 2002). Cyclins and CDKs form heterodimeric CYC/CDK complexes with at least some of the substrate specificity conferred by the cyclin partner (Loog and Morgan 2005). In yeast, a single CDK binds to different cyclins to regulate the cell cycle progression, whereas in plants and mammals multiple CDKs as well as multiple cyclins function at different stages of the cell cycle (Morgan 1997; Mendenhall and Hodge 1998; Wang et al. 2004).

Despite having the same core cell cycle mechanism as other eukaryotes, the plant cell cycle has some unique features. For example, plants have a unique class of plant-specific CDKs, the CDKBs (Porceddu et al. 2001; Vandepoele et al. 2002; Boudolf et al. 2006). Unlike in animal CDKs and plant CDKA;1, these CDKBs do not complement *cdc2/cdc28* yeast mutants (Imajuku et al. 1992; Fobert et al. 1996)**.** The CDKBs consist of two subfamilies, with expression of CDKB1 starting during S phase and peaking in G2 and CDKB2 expression starting later and peaking at the G2 to M transition (Menges et al. 2005). Unlike in mammals, CDK phosphorylation and de-phosphorylation does not require WEE1 kinase and CDC25 phosphatase for inhibition and activation of CDK kinase activity, respectively (Boudolf et al. 2006; Perry and

Kornbluth 2007). Plants lack a CDC25 ortholog, and WEE1 is involved in the DNA damage response rather than playing a significant role in the normal mitotic cell cycle in *Arabidopsis* (De Schutter et al. 2007; Dissmeyer et al. 2009; Dissmeyer et al. 2010). Therefore, the G2/M transition in plants appears to be controlled primarily by cell cycle phase-specific transcription of CDKB1s, CDKB2s and their cyclin partners, rather than by the WEE1 kinase/CDC25 phosphatase pair that regulates entry into M-phase in animals and some fungi (Boudolf et al. 2006).

In plants, two CDK inhibitor (CKI) families have been reported. One is the INHIBITOR/INTERACTOR OF CYCLIN-DEPENDENT KINASES/KIP-RELATED PROTEIN (ICK/KRP) family, named because of their sequence similarity with animal Kip-type CDK Inhibitors (Wang et al. 1997; De Veylder et al. 2001). *Arabidopis* has seven *ICK/KRP* family members: *ICK1/KRP1, ICK2/KRP2, KRP3, KRP4, KRP5, KRP6,* and *KRP7* (De Veylder et al. 2001; Wang et al. 2008). KRPs are direct inhibitors of CDKA;1 kinase activity and when expressed at high level can result in cell death (Schnittger et al. 2003; Verkest et al. 2005a; Jun et al. 2013; Wen et al. 2013). A second plant CDK inhibitor family is encoded by the *SIAMESE-RELATED* (*SMR*) genes (Churchman et al. 2006). *SIAMESE* is the founding member of the family, and other *Arabidopsis SMR*s have been implicated in binding to several different CYC/CDK complexes, and the rice SMR protein EL2 has been shown to inhibit CDKA;1 kinase activity (Churchman et al. 2006; Peres et al. 2007; Van Leene et al. 2010).

Both *SIM* and the closely related *SMR1/LGO* gene were both discovered via their role in promoting a modified version of the cell cycle known as endoreplication in *Arabidopsis* trichomes (shoot epidermal hairs) and sepal epidermal cells, respectively. In the case of *sim* mutants, the result is that the trichomes, which are unicellular in wild-type plants, divide and

become multicellular (Walker et al. 2000; Churchman et al. 2006). During endoreplication, also known as endoreduplication, DNA replicates without subsequent mitosis and cytokinesis, consequently doubling the DNA amount in each round of the cell cycle, resulting in cells with increased ploidy (Edgar and Orr-Weaver 2001; De Veylder et al. 2011; Fox and Duronio 2013). In plants, endoreplication occurs in tissues that develop mass very quickly or have a higher metabolic rate, and is often correlated with cell differentiation and increased cell size.

Recently, additional roles for *SMRs* in plant growth and development have been identified. Several *SMRs* have been implicated in regulating root meristem size in response to gibberellin signaling, although this link has not been conclusively demonstrated (Achard et al. 2009). Additionally, *SMR5* and *SMR7* expression inhibits mitotic division in response to DNA damage (Yi et al. 2014). *SIM* and *SMR1* have also been implicated as inhibitors of CDK activity associated with plant response to pathogens (Wang et al. 2014).

Multiple lines of evidence have identified two types of CYC/CDK complex, CYCD3/CDKA;1 and CYCA2;3/CDKB1;1, as important negative regulators of endoreplication (Dewitte et al. 2007; Boudolf et al. 2009; De Veylder et al. 2011). Constitutive overexpression of *CYCD3;1*, a well-established CDKA;1 partner, strongly reduces endoreplication and increases cell proliferation in *Arabidopsis* leaves, whereas loss of *CYCD3* function in *cycd3;1-3* triple mutants lacking functional copies of all three *CYCD3* genes results in increased endoreplication in *Arabidopsis* sepals and leaves (Dewitte et al. 2003; Dewitte et al. 2007). Additionally, ectopic expression of *CYCD3;1* in trichomes results in cell divisions, phenocopying the *sim* mutant multicellular trichome phenotype (Schnittger et al. 2002; Dewitte et al. 2003). Loss of function of *CYCA2;3*, a interacting partner of CDKB1;1, increases ploidy level and overexpression inhibits endoreplication in the *Arabidopsis* leaves (Yu et al. 2003; Imai et al. 2006; Boudolf et al.

2009). In addition, overexpression of a *CDKB1;1* dominant-negative allele leads to increased endoreplication in the *Arabidopsis* leaves, which indicates that CDKB1;1 restrains endoreplication and promotes progression into mitosis (Porceddu et al. 2001; Boudolf et al. 2004).

Although substantial evidence indicates that SIM and SMRs are CKIs, it is not clear yet which specific CYC/CDK complexes are inhibited by SIM to promote endoreplication in *Arabidopis* developing trichomes. Interaction of both SIM and a rice homolog (EL2) with D-type cyclins and CDKA;1, and not with CDKBs or mitotic cyclins, in Förster Resonant Energy Transfer (FRET) experiments in vivo, as well as inhibition of CDKA;1 kinase activity by the rice SMR EL2 indicates that SIM and other SMRs are likely inhibitors of CYCD/CDKA;1 complexes (Churchman et al. 2006; Peres et al. 2007). On the contrary, co-purification from plant cells of SIM, SMR1 and SMR2 with CDKB1;1, and not with CDKA;1, in affinity-tagging experiments suggests that SIM may primarily interact with and inhibit CDKB1;1 kinase complexes (Van Leene et al. 2010). In the same study, SIM and SMR11 were found to interact with CYCB2;4, an interacting partner of CDKB1;1 (Van Leene et al. 2010), while other SMRs were found to interact with CYCDs and CDKA;1. On the basis of these latter results, it has been suggested that the SIM/SMR family members are divided into two functionally distinct groups, with SIM, SMR1, SMR2 and SMR11 being inhibitors of CDKB1;1 complexes, and the remaining SMRs being inhibitors of CDKA;1 complexes (Van Leene et al. 2010; Yi et al. 2014). Although multiple *SMR* genes are present in the genomes of most plants examined so far, functions are only known only for the few *Arabidopsis SMR*s noted above, and little is known about the functions of *SMR*s of other plants.

In this study, we show that the SIM/SMR family members are functionally conserved throughout land plant evolution, and that even divergent members of the family can replace *SIM* function in vivo. We also present evidence from both in vitro and in vivo studies that inhibition of CYCD3/CDKA;1 complexes is important to SIM function, and demonstrate a novel role for *SMR2* in regulating the rate of cell proliferation. Our work suggests that differences in SMR function in plant growth and development are primarily due to differences in transcriptional and post-transcriptional regulation, rather than differences in fundamental biochemical function.

3.2 Divergent members of the SMR family can functionally replace SIM

The initial paper describing *SIM* also described three related *SMR* genes from the *Arabidopsis thaliana* genome, as well as several *SMR*s from a variety of dicots and monocots (Churchman et al. 2006), and two subsequent papers described three more classes of *SMR*s in *Arabidopsis* (Peres et al. 2007; Yi et al. 2014). These members of the *SMR* family were described based on a series of short conserved protein sequence motifs that occurred in a consistent order, but with variable spacing. Several other *Arabidopsis SMR*s have been referred to in the literature (Van Leene et al. 2010; Yi et al. 2014), but the sequence similarities among these genes have not been described, and the functions of most of these genes as cell cycle regulators have not been tested. This is important in light of the low level of sequence conservation in the family, and the suggestion by Van Leene et al. (2010) that different SMRs may inhibit different CYC/CDK complexes.

An exhaustive iterative search of the *Arabidopsis thaliana* genome revealed a total of 17 putative *SMR* genes (*SIM* and *SMR1-16*), and a consensus sequence for three conserved protein sequence motifs was derived from these genes (Figure. 3.1A). Although most of these *SMR*s

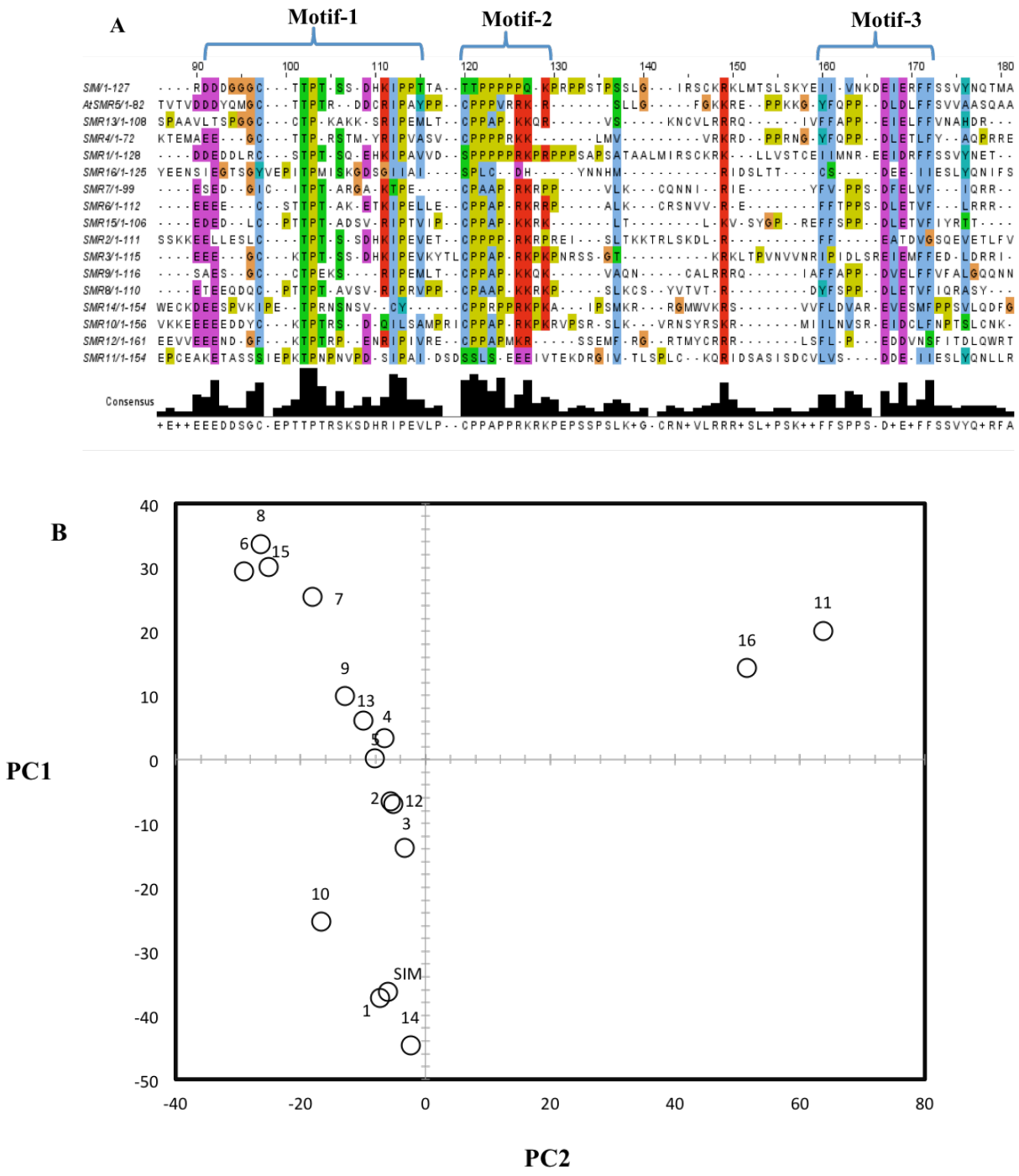


Figure 3.1. Alignment of *Arabidopsis thaliana* SMRs (A) showing three most conserved motifs (1, 2 and 3) among SMRs. (B) Principle Component Analysis (PCA) indicating that SMR11 and 16 are the most divergent members of the SIM/SMR family in *Arabidopsis*.

were readily recognizable as being related to *SMR*s with known functions, other putative members of the family were more divergent. We had previously shown that the rice *SMR* known as *EL2* could complement the multicellular trichome phenotype of *sim* mutant plants, in spite of having only limited sequence similarity (Peres et al. 2007).

For this reason, we tested whether a wide variety of *Arabidopsis SMR*s could complement *sim* when expressed under the control of the *GL2* promoter, which in leaves is relatively trichome specific. We found that *SMR2*, *SMR4* and *SMR11* as well as *SMR3, SMR7* and *SMR13* (Figure 3.2, Figure 3.3), could all complement the *sim* phenotype efficiently, indicating that all of these genes are able to functionally replace *SIM* in suppressing mitosis and promoting endoreplication in developing trichomes. In spite of its ability to complement *sim*, a principal component analysis in which components were generated by pairwise peptide sequence alignments to represent spatial similarities based on a substitution matrix showed that *SMR11* and *SMR16* together and are clearly distinct from the rest of the *SMR*s (Fig. 3.1B).

For *SMR5*, attempts to complement *sim* using the annotated ORF that is available on The Arabidopsis Information Resource (TAIR) website, with an annotated intron removed, were unsuccessful (Figure 3.3 F). We noticed that the predicted protein translated from an unspliced transcript was a better match to the SMR motif, so we transformed unspliced SMR5 in the *sim* background plants and observed that unspliced SMR5 complemented *sim* mutant trichome (Figure 3.2 E, F).

3.3 SMRs are conserved in all major land plant lineages

We next conducted a systematic search for all *SMR*-like genes in the sequenced genomes of the dicots *Carica papaya* L., *Citrus* x *sinensis* (L.) Osbeck, and *Eucalyptus grandis* W.Hill ex Maiden, the monocot *Oryza sativa* L., the lycophyte *Selaginella moellendorffii*, and the

bryophyte *Physcomitrella patens* (Hedw.) Bruch & Schimp. Each of these genomes contained multiple *SMR*-like sequences (Figure 3.4B), while no putative *SMR*s were detected in the genomes of the algal species *Chlamydomonas reinhardtii* or *Ostreococcus tauri*. The 85 putative land plant *SMR* genes that we have identified were used to build a neighbor-joining tree (Fig. 3.4B).

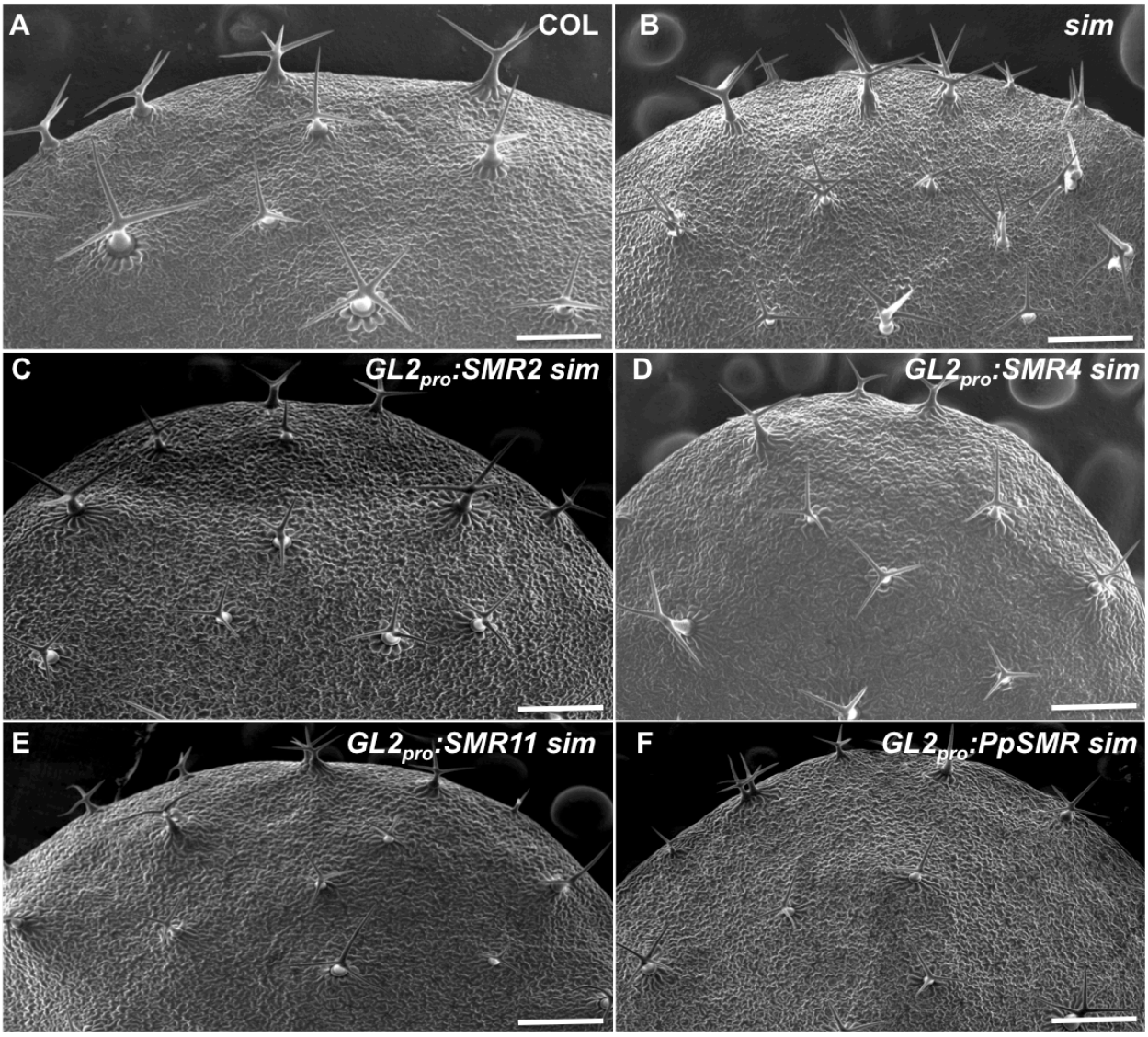


Figure 3.2 Different SMRs are complementing multicellular *sim* mutant trichomes (A) Wild-type trichome (B) Multicellular *sim* mutant trichomes (C) SMR2 (D) SMR4 (E) SMR11, one of the most diverse *SMR* among *Arabidopsis SMRs*, and (F) Phylogenetically most diverse PpSMR (*Physcomitrella patent,* a moss) also restored unicellular wild-type trichome. In all images scale bars are 200μm.

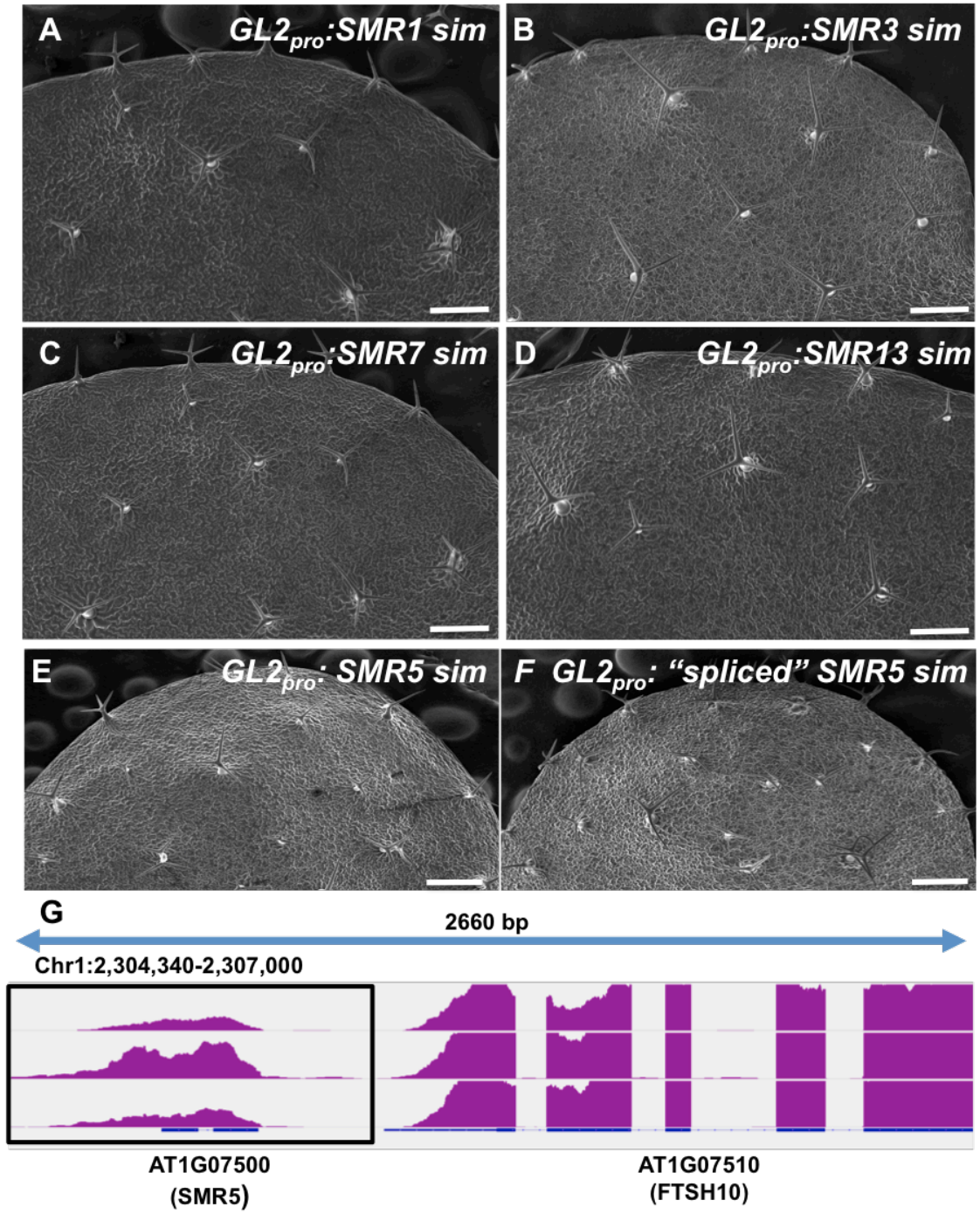


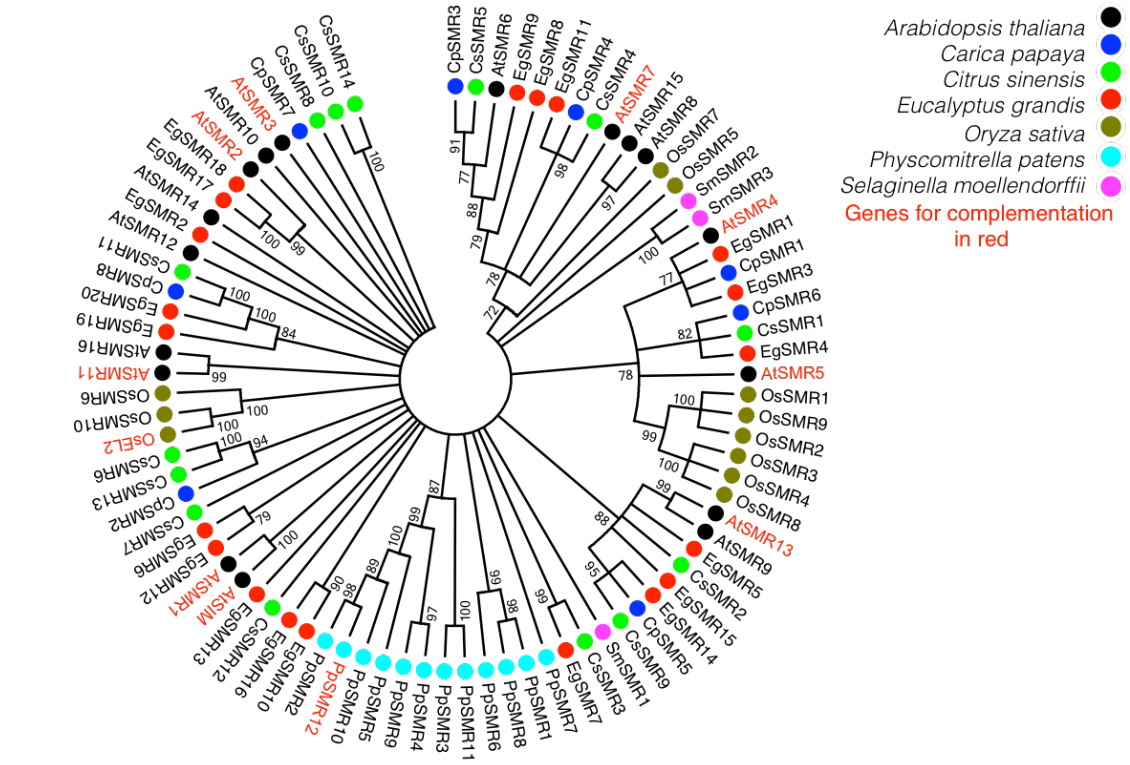
Figure 3.3 More Arabidopsis SMRs complementing *sim* mutant trichomes. (A) SMR1 (B) SMR3 (C) SMR7 (D) SMR13, and (E) SMR5 complemented, whereas (F) "Spliced" SMR5 was unable to complement *sim* mutant trichome. (G) On left side, RNA seq data shows that SMR5 does not have any intron, on right side an internal control showing both intron and exon. SMR5 and internal control three lanes are three different samples. Scale bars are 200 μm.

Although the short length of the conserved region of *SMR*s, combined with relatively low sequence conservation, leaves the relationships among many of these genes unresolved, a number of clades with strong bootstrap support are evident. Of particular note, the *Arabidopsis SMR*s that we have shown to complement *sim* include genes in all major clades and these clades include genes from other angiosperms as well. *AtSMR*s are indicated by black dots, genes used for complementation are labeled in red (Figure 3.4A). The bryophyte *Physcomitrella patens* is the most phylogenetically divergent lineage for which we have obtained putative *SMR* sequences. We chose one putative *Physcomitrella SMR* (*PpSMR* in Figure 3.4) that was in a well-supported *Physcomitrella*-specific clade, and tested it for its ability to complement the *Arabidopsis sim* mutation. As shown in Figure 3.2F, *PpSMR* readily complements *sim*, indicating that this phylogenetically distant *SMR* is functionally equivalent to *SIM*. Combined with the observation that the rice gene *OsEL2*, previously shown to complement *sim* (Peres et al. 2007), is located in a rice-specific clade quite far from *SIM* in the gene phylogeny, our results suggest that most or all of the putative *SMR*s we have identified encode cell cycle regulators that are functionally equivalent to *SIM*.

3.4 SIM interacts with both CDKA;1 and CDKB1;1 in *Arabidopsis* protoplasts.

As noted above, previous studies have reported conflicting results with regard to whether SIM interacts primarily with CDKA;1 or with CDKB1;1 (Churchman et al. 2006; Peres et al. 2007; Van Leene et al. 2010). Therefore, protein-protein interaction experiment results are not consistent and it remains unclear that which CDK interacts with SIM. For this reason, we have used another protein-protein interaction assay, the split-luciferase complementation assay, to test interactions between SIM and CDKs in *Arabidopsis* protoplasts (Fujikawa and Kato 2007).

(A)



(B)

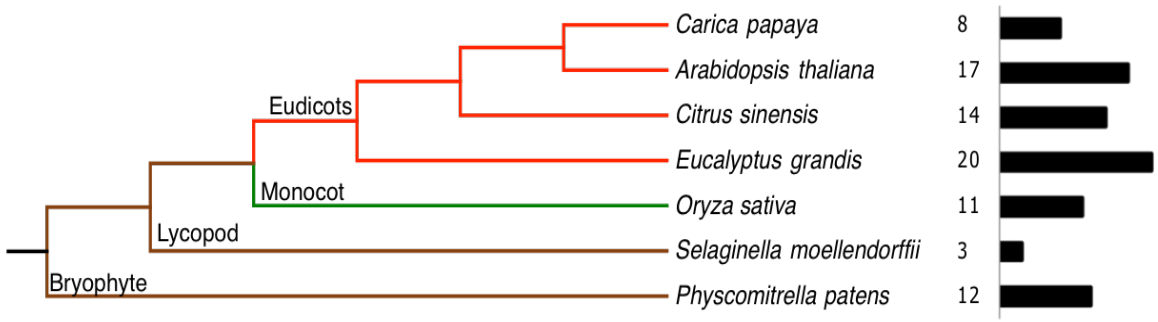


Figure 3.4 Different SMRs of *Arabidopsis* and phylogenetically most divergent *Physcomitrella patens (PpSMR)* complement *sim* mutant (A) Phylogenetic tree showing that *SMRs* from *Arabidopsis*, rice and even most divergent *Physcomitrella patens* (PpSMR) complemented (in red) *sim* indicating that all SMR family members likely have same functional mechanism as the SIM protein (B) SMR phylogenic tree on the basis of different land plants SMR sequence similarity, showing *Arabidopsis* and *Physcomitrella patens* are most distantly related.

Because split-protein complementation assays are dependent on the geometry of the interacting proteins, both *SIM* and *CDKA;1* were fused to the two the N-terminal (Nluc) and C-terminal (Cluc) halves of *Renilla* luciferase in all eight possible fusion combinations and tested for functional luciferase activity in *Arabidopsis* protoplasts (Fig.3.5).

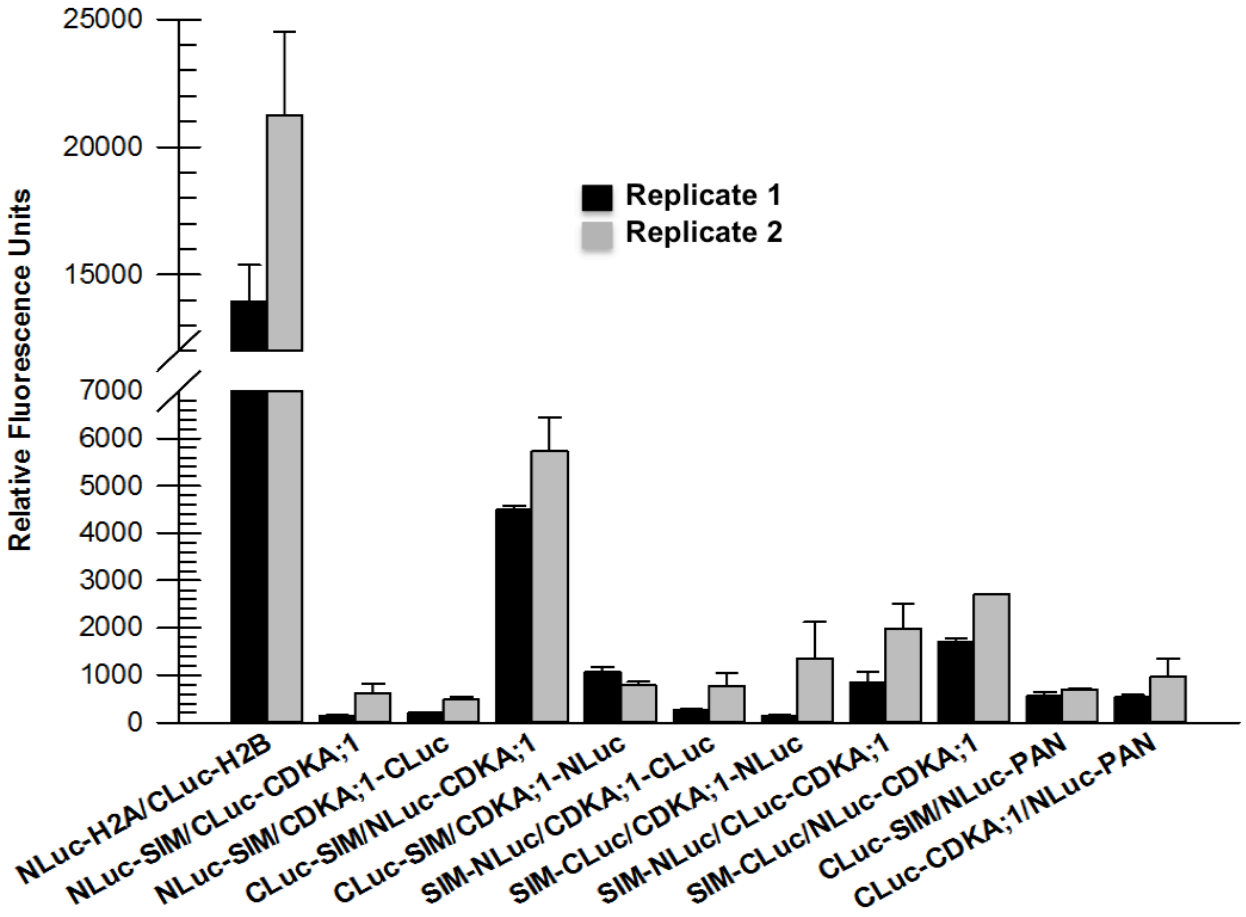


Figure 3.5 All eight possible combinations of SIM and CDKA;1 showing that Cluc-SIM and Nluc-CDKA;1 is the best combination in Split Luciferase Complementation (SLC). Histone2A and Histone2B, and transcription factor PARIANTHIA served as positive and negative controls respectively.

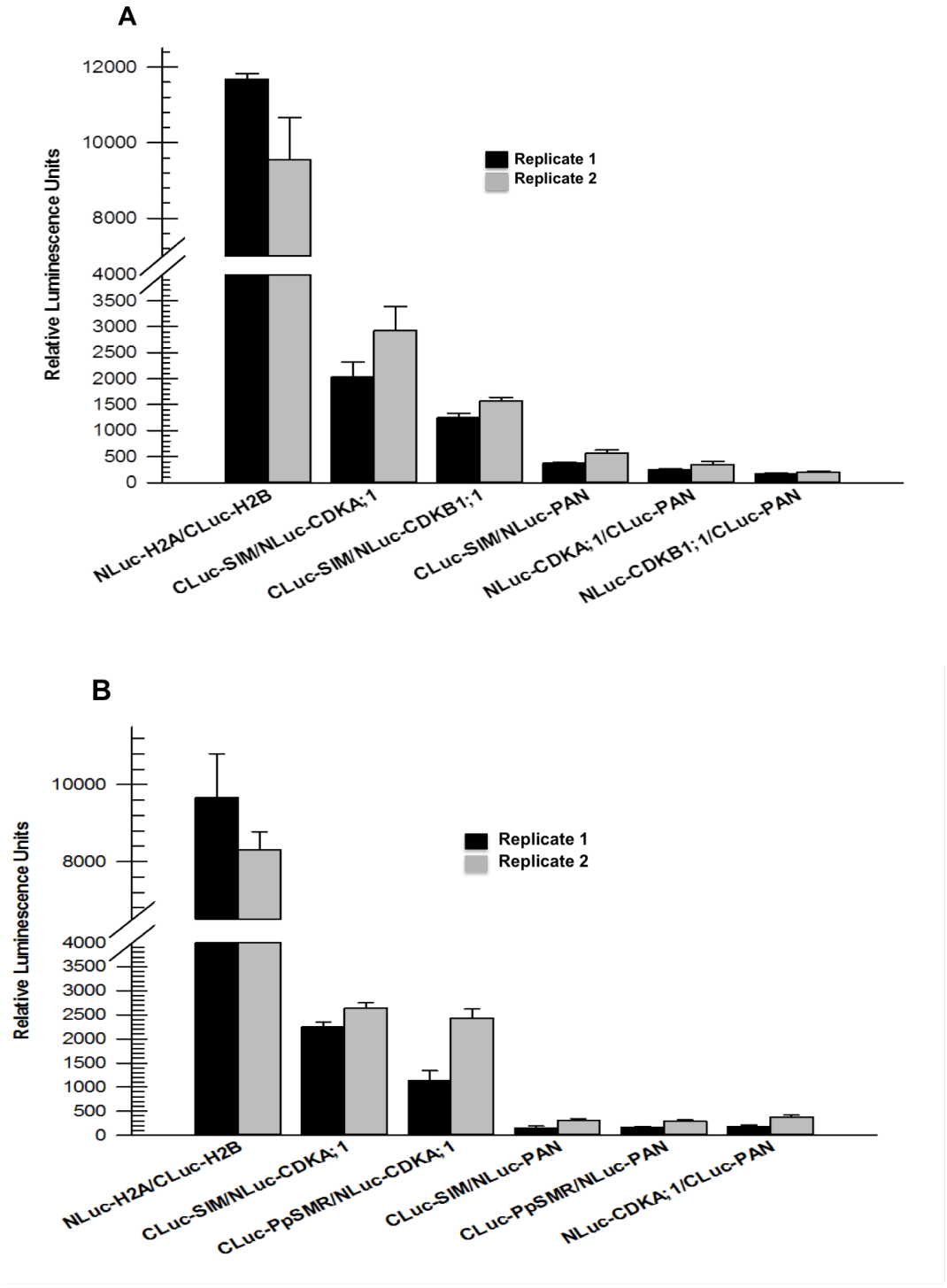


Figure 3.6. SIM and *Physcomitrella patent* (PpSMR) interact with CDKA;1 in Split Luciferase Complementation (SLC) assay. (A) SIM interacts with both CDKA;1 and CDKB1;1 (B) Phylogenetically diverse *Physcomitrella patens* (PpSMR) also interacts with CDKA;1. Histone2A and Histone2B (H2A-H2B), and PARIANTHIA are positive and negative control, respectively.

Although several combinations gave activity above that of negative controls, the orientation giving the strongest signal, with Cluc at N-terminus of SIM and Nluc at N-terminus of CDKA;1, was used for all other tests of SIM-CDK interactions (Fig. 3.5). Interaction of Histones H2A and H2B was used as a positive control and interaction with the transcription factor PERIANTHIA was used as a negative control. In this assay, we observed that SIM reproducibly interacted with both CDKA;1 and CDKB1;1 (Fig. 3.6A). The phylogenetically distant *Physcomitrella patens PpSMR*, used above for complementation, also interacted with CDKA;1 (Fig. 3.6B).

3.5 SIM inhibits the activity of both CDKA;1 and CDKB1;1 in vitro

The interaction of SIM with both CDK;1 and CDKB1 suggested that SIM may inhibit the kinase activity of both CDKs. Evidence for the direct inhibition of CDK kinase activity by SIM/SMRs family members has only been demonstrated is only SIM homolog in rice SMR EL2 (Peres et al. 2007). For this reason, we tested the ability of SIM to inhibit kinase activity of various cyclin/CDK complexes, using a recently described in vitro CDK assay system (Harashima and Schnittger 2012). The SIM protein proved difficult to express in *E. coli*; His-tagged SIM was largely insoluble, and we were unable to completely remove co-purifying contaminants from Glutathione-S-transferase (GST)-tagged SIM, which was used for the assays presented here. This partially purified GST:SIM inhibits kinase activity of CYCD3;1/CDKA;1, CYCD2;1/CDKA;1 and CYCB1;1/CDKB1;1 complexes in a dose-dependent manner, while a control GST:GFP fusion protein did not inhibit these complexes (Figure 3.7 A, B and C), The GST:SIM band appears to be phosphorylated by CYCB1;1/CDKB1;1 (Figure 3.7C, **) and not by the other kinases, although CYCB1;1/CDKB1;1 also appears to phosphorylate GST:GFP (Figure 3.7C, *). *Physcomitrella* PpSMR also inhibited CDKA;1/CYCD3;1 kinase activity (Figure 3.7D).

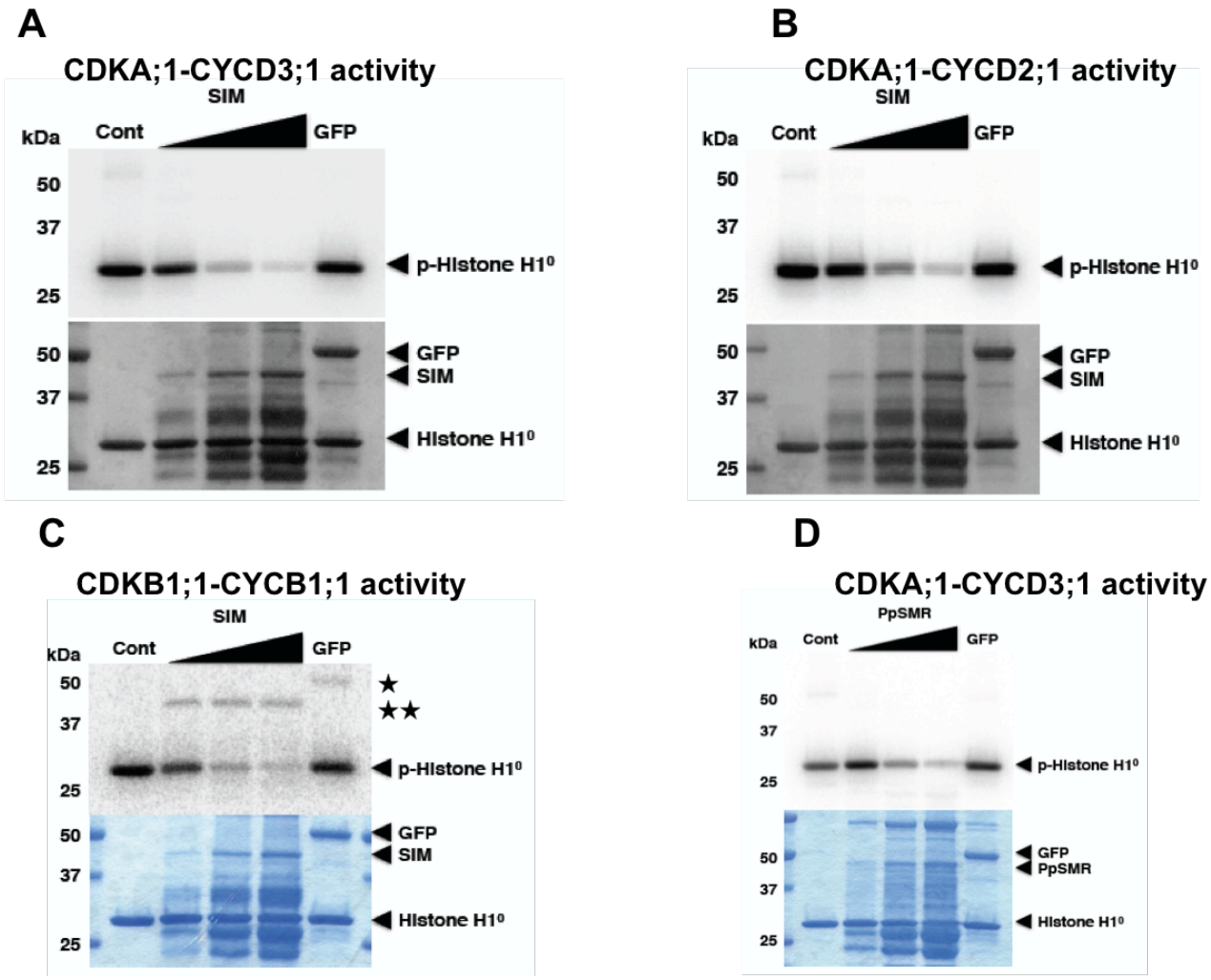


Figure 3.7 SIAMESE (SIM) and *Physcomitrella patent* (PpSMR) inhibits CYC/CDKs kinase activity in a dose dependent manner. (A) CDKA1;1/CYCD3;1 (B) CDKA;1/CYCD2;1, and (C) CDKB1;1/CYCB1;1 kinase activity is inhibited by SIM, and (D) CDKA;1/CDYD3;1 kinase activity is also inhibited by PpSMR. GFP, a negative control, is showing no inhibition of kinase activity. All four panels have both kinase assay (top) and stained gel (lower).

3.6 Cell division in *sim* mutant trichomes depends upon the function of both CYCD3s and CDKB1

Our in-vitro results (Figure 3.7) suggest that SIM inhibits the CYC/CDKs complexes, so we predicts that cell division in *sim* mutant trichomes should depend on the function of one or both of these *CDK*s. D3-type cyclins are generally thought to be activators of CDKA;1

complexes (Dewitte et al. 2003; Menges et al. 2006; Dewitte et al. 2007; Van Leene et al. 2010), and CYCD3;1 is only capable of activating

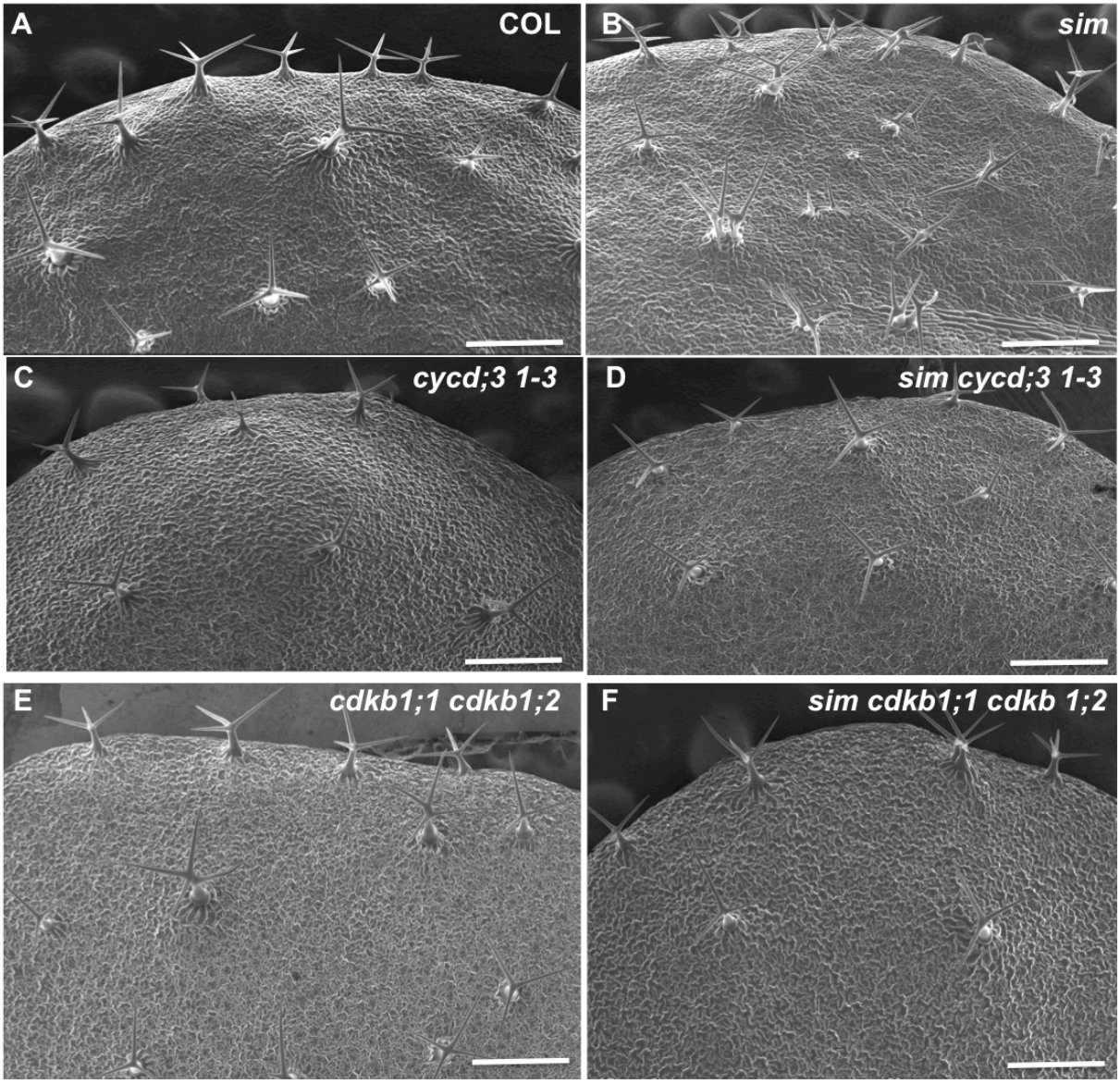


Figure 3.8 SIM restrains the activity of CYCD3 and CDKB1 in trichome development.
(A) Wild-type Trichome (B) Multicellular *sim* trichomes (C) Loss of function mutation of *cycd3,1-3* in COL plants (D) Mutation all *cycd3, 1-3* in *sim* plant restored wild-type plants. (E) *cdkb1;1-cdkb1;2* mutation in COL plant does not effect trichome phenotype, whereas (F) *cdkb1;1-cdkb1;2* restore wild-trichome in *sim* mutant plants. All scale bars are 200μm.

CDKA;1 in vitro, not CDKB1;1 (Nowack et al. 2012). Triple mutants lacking all three D3-type cyclins (*cycd3;1-3*) produce unicellular trichomes resembling those of wild-type. We constructed *sim cycd3;1-3* quadruple mutants, and they exhibited no cell division in trichomes, indicating that the *cycd;1-3* phenotype is epistatic to the *sim* division phenotype (Figure 3.8 C, D).

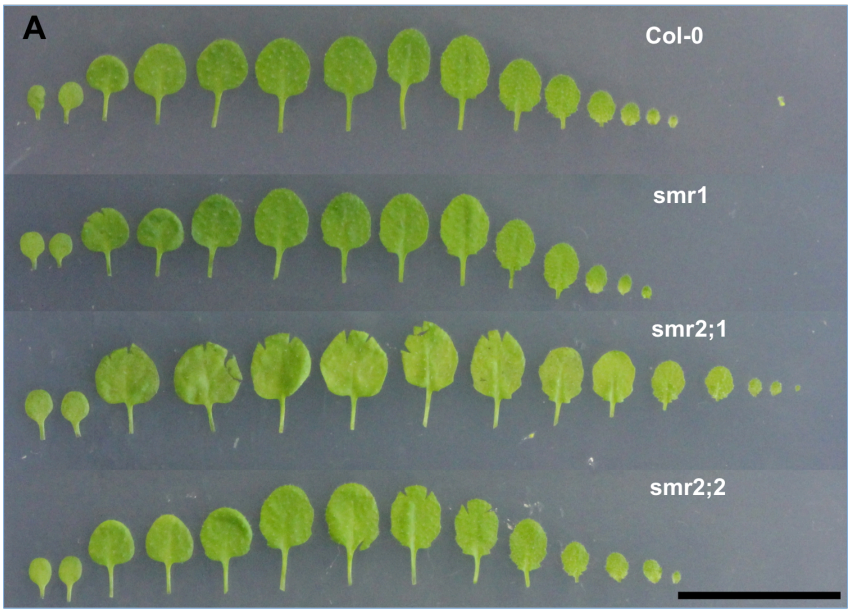
Table 3.1 Both CYCD3 and CDKB1 are necessary for cell division in *sim* mutant trichome. The numbers of DAPI-stained trichome nuclei at each trichome initiation site (TIS) were counted for each genotype.

Genotype	Number of Nuclei per TIS	Number of TIS/Total No of trichome
Col-0	1.0±0.00	60
Sim	2.46±1.41	60
cycd3;1-3	1.0±0.00	60
sim cycd3;1-3	1.10±0.30	60
cdkb1;1 cdkb1;2	1.0±0.00	60
sim cdkb1;1 cdkb1;2	1.25±0.44	60

Similarly, *cdkb1;1 cdkb1;2* double mutant plants have wild-type trichomes showing no division (Figure 3.8 E). Homozygous *cdkb1;1/cdkb1;2/sim* plants were identified. Homozygous *sim cdkb1;1 cdkb1;2* triple mutants were also primarily unicellular (Figure 3.8F, Table 3.1) indicating cell division in *sim* mutant trichomes is also dependent upon CDKB1 activity.

3.7 SMR2 restricts cell proliferation and co-operates with SIM and SMR1 to promote endoreplication during leaf development.

In the course of this work, we identified T-DNA insertion alleles of several *Arabidopsis SMR*s, including *SMR1* and *SMR2*. We noticed that both *smr2* alleles identified had larger leaves



B

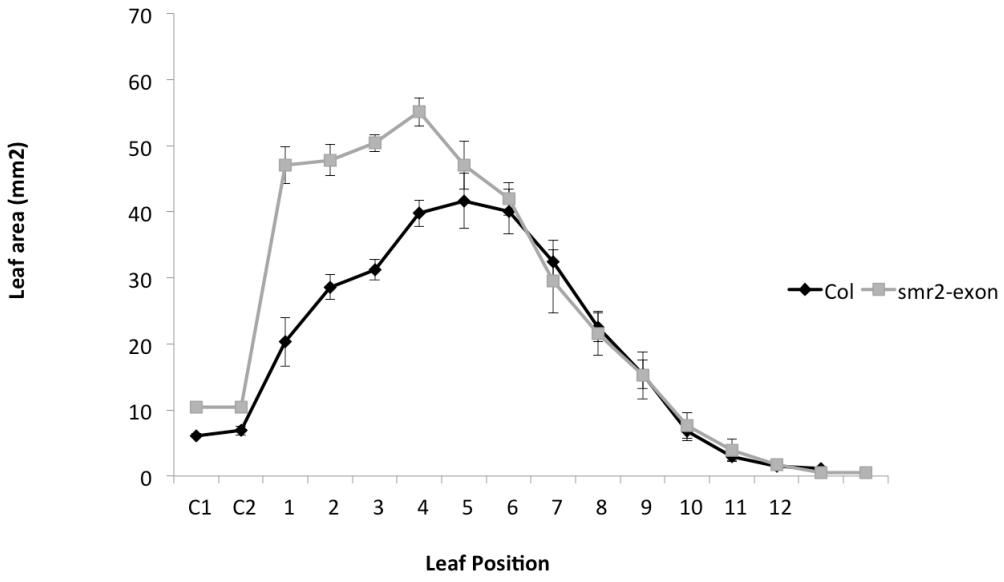


Figure 3.9 Functional loss of *smr2-1* leads to increase in *Arabidopsis* leaf size.

(A) Functional loss of *smr2* plants have bigger leaves than COL and *smr1* mutant plant (B) Insertion in *smr2* exon shows bigger leaves.

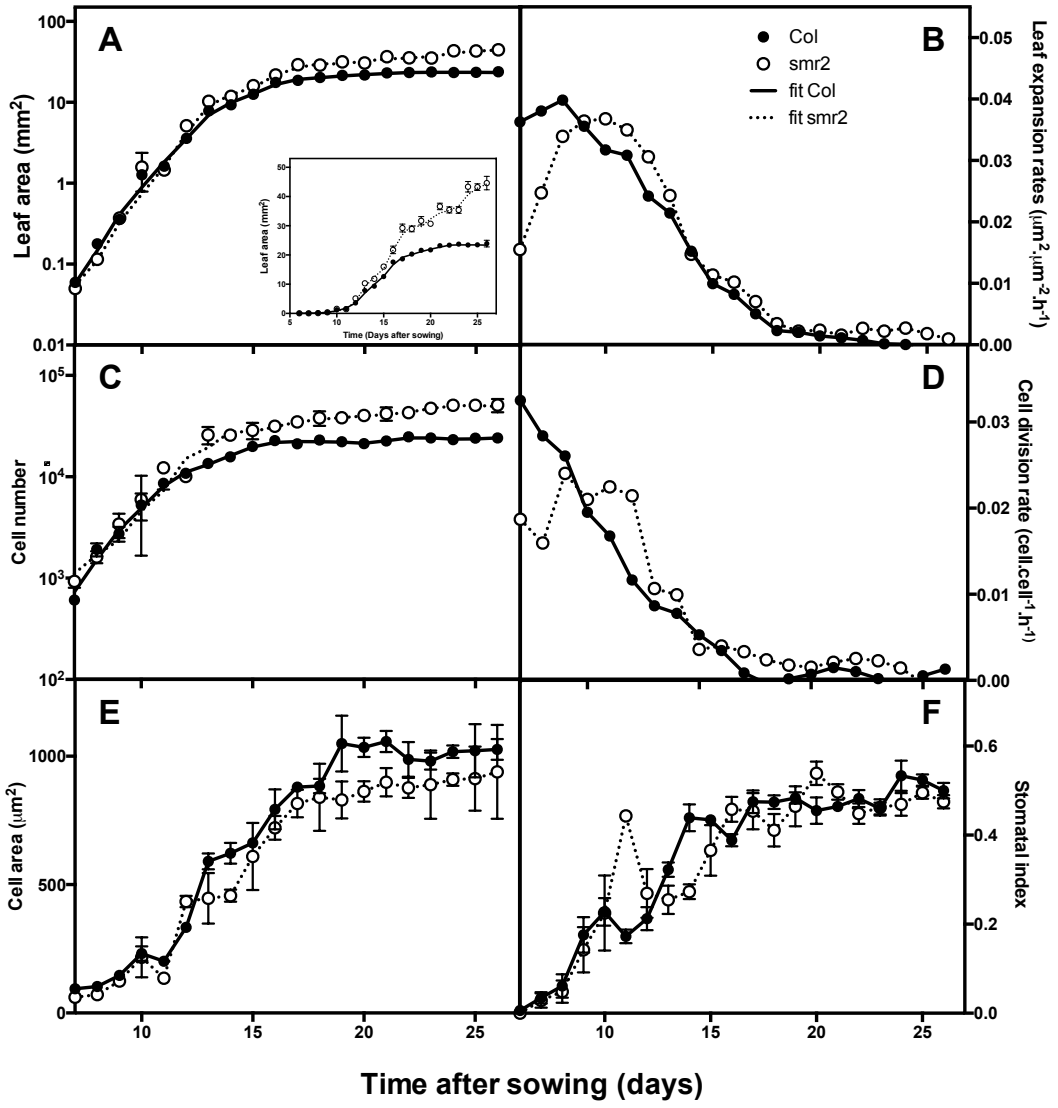


Figure 3.10 SMR2 restricts cell proliferation and co-operates with SIM and SMR1 to promote endoreplication in leaf development.

than wild-type, particularly on the first six leaves; this phenotype was noticeably stronger in the *smr2-1* allele, which has an insertion in the sole exon of the gene (Figure 3.9 A, B). This allele produces no detectable *SMR2* transcript (Peres et al. 2007).

A kinematic analysis was conducted comparing the parameters of leaf growth on first leaves of wild-type and *smr2-1* mutant plants. An increase in leaf cell number was apparent

starting at 10 days after sowing (10 DAS, Figure 3.10 C). This corresponds to a notable increase in cell division rate at 10-12 DAS; at 12 DAS the cell division rate of the mutant was approximately double that of wild-type (Figure 3.10D). In contrast, average cell area of mutant leaves by the end of the time period investigated (26 DAS) was only slightly less than wild-type, indicating that the cause of increased mutant leaf size was primarily due to increased cell proliferation. Endoreplication levels were also monitored by flow cytometry throughout leaf development; while the time of appearance of 4C, 8C and 16C cells in mutant leaves were delayed, the mutant leaves ultimately reached ploidy levels similar to those of wild-type leaves. We also examined the ploidy of leaf cells of *sim* and *smr1* single mutants, as well as a *sim smr1 smr2* triple mutant. Although the individual mutants have little effect on the endoreplication index in leaves (the average number of endocycles per cell), leaf cells of the triple mutant exhibited a strongly reduced DNA content and underwent a reduced number of endocycles (Personal communication with Dr. Briemstee)

3.8 Discussion

In the work presented here, we have identified three previously undescribed putative *Arabidopsis SMR* genes, *AtSMR14*, *AtSMR15* and *AtSMR16*, and have identified a total of 85 putative *SMR*s in the sequenced genomes of seven plant species representing a wide range of land plant lineages. Most of these genes contain all three of the key protein sequence motifs defining the SMR family (Figure 3.1A), originally identified as Motifs-1, 2 and 4 by Churchman et al. 2006. Motif-1 contains a threonine followed by a proline, which is the minimal consensus site for phosphorylation by CDKs. This pair of amino acids is the most highly conserved throughout *SMR* evolution, suggesting that phosphorylation of this threonine may be crucial to SMR function. Motif-2 is proline-rich, and typically contains sequences of the form PXXP,

followed by one or more basic residues. This resembles protein interaction domains that interact with partner proteins by forming a PP II helix (Kay et al. 2000). Motif-4 has previously been reported to be a cyclin-interaction domain in the rice EL2 protein (Peres et al. 2007).

Although all three of these motifs occur in the same order, but with variable spacing in most *SMR*s, some *SMR*s are exceptions to this pattern. *AtSMR11* and *AtSMR16* for example, lack a detectable Motif-2, although in spite of this sequence divergence, *AtSMR11* was able to complement a *sim* mutation (Figure 3.2E). *AtSMR3*, which also complemented *sim* (Figure 3.3B), appears to contain a second copy of Motif-1 located between Motif-2 and Motif-3 (residues 82 to 91, TPVNVVNRIP). Finally, a phylogenetically distant *SMR* from the bryophyte *Physcomitrella patens* also complemented the *sim* mutant phenotype (Figure 3.2F). Taken together, these results indicate that most, if not all, of the *SMR* sequences that we have identified represent same code for functional SMR proteins. These proteins appear to be functionally equivalent, based on their ability to complement the *sim* trichome phenotype. Thus the roles played in plant development by different *SMR*s are most likely maintained by location in transcriptional and posttranscriptional regulation of individual genes, rather than by differences in their underlying biochemical activity.

Consistent with the overlapping functional roles among *SMR*s, we have found that *SIM*, *SMR1*, and *SMR2* cooperate to promote endoreplication during leaf development. The three single mutants affect endoreplication levels only marginally, but the degree of endoreplication is greatly reduced in the triple mutant. Only the *smr2* mutant shows an increase in leaf size (Figure 3.10A) and leaf cell number (Fig. 3.10 C), due to a briefly increased rate of cell division in the mutant at day 10-12 (Fig. 3.10C), approximately the time when endoreplication is first established in these leaves (Beemster et al. 2006). Although endoreplication in *smr2* mutants lags

behind wild-type initially, it ultimately reaches the same level. Both *SMR1* and *SMR2* were shown to complement *sim* (Figure 3.3A, figure 3.2C) and thus these three *SMR*s encode similar functions. It may be that *SMR2* plays a role in initiating endoreplication in the leaf, and that *SIM* and *SMR1* are necessary for maintenance of endocycles and/or controlling the final extent of endocycling.

In spite of earlier results suggesting that SIM specifically interacted with either CDKA;1 (Churchman et al. 2006) or CBKB1;1 (Van Leene et al. 2010), the results presented here suggest that SIM interacts with (Figure 3.6A), and inhibits the activity of both CDKA;1 and CDKB1;1 kinase complexes (Figure 3.7). Furthermore, SIM is capable of inhibiting the kinase activity of both CYCD3;1-containing and CYCD2;1-containing CDKA;1 complexes (Figure 3.7 A, B).

That SIM is capable of inhibiting such a broad range of CDK complexes, both CDKA;1 complexes typically thought of as G1/S kinases, and CDKB1;1 complexes typically thought of as G2/M kinases, raises the question of why SIM inhibits only mitosis, and not S-phase. KRPs inhibit a similar broad range of CDK complexes, and depending on their level of expression can inhibit mitosis only, triggering endoreplication, or can completely block entry into S-phase when expressed in trichomes, ultimately resulting in cell death (Schnittger et al. 2003). Posttranscriptional regulation by cell cycle stage-specific phosphorylation or protein degradation, limiting active SIM CDK inhibitory activity to G2/M provides one possible explanation. Epistasis of *cycd3* and *cdkb1* mutants to *sim* shows that cell division in *sim* mutant trichomes depends upon both CYCD3 and CDKB1 function. This in vivo result is consistent with the implications of our interaction and inhibition studies, suggesting that both CYCD3;1/CDKA;1 and CDKB1-containing complexes may be direct targets of SIM inhibition in vivo.

The involvement of D3-type cyclins in the regulation of mitosis in this context is particularly intriguing. CYCD3s have generally been considered to be G1/S cyclins. In vitro, CYCD3;1 can activate the kinase activity of CDKA;1, considered to be the main G1/S CDK, and not the kinase activity of the mitotic CDK CDKB1;1, and CYCD3;1/CDKA;1 complexes can phosphorylate RBR, the gatekeeper of the G1/S checkpoint (Nowack et al. 2012). In cell culture, *CYCD3;1* overexpression promotes the G1/S transition, and cells accumulate in G2, consistent with a primary role in regulating entry into S-phase (Menges et al. 2006). In contrast, within six hours of induction of CYCD3;1 in *Arabidopsis* seedlings, expression of a group of genes directly related to mitosis is significantly up regulated, suggesting that CYCD3-containg CDK complexes may play a role in promoting progression through mitosis. These mitotic genes are not upregulated by induction of either E2Fa or E2Fc, key transcription factors downstream from RBR in the G1/S checkpoint. Elimination of *CYCD3* function in a triple mutant defective for all three D3-type cyclin genes (the same triple mutant used in this study) results in increased endoreplication, indicating that *CYCD3*s suppress endoreplication in the leaf (Dewitte et al. 2007). Finally, and most telling for our work, ectopic expression of *CYCD3;1* in developing trichomes causes the trichomes to divide, phenocopying the *sim* mutant phenotype, while ectopic expression of CYCD2;1 has no effect (Schnittger et al. 2002). Recent work indicates that CYCD3 is part of a complex web of interactions involving RBR and multiple E2F transcription factors that control the balance between cell division and endoreplication (Magyar et al. 2012). Our work points directly toward CYCD3/CDKA;1 complexes as targets of inhibition by SIM and other SMRs to inhibit mitosis and promote endoreplication.

CHAPTER 4
ANALYSIS OF THE PHOSPHORYLATION AND SUBCELLULAR LOCALIZATION CONTROL OF THE *ARABIDOPSIS* CYCLIN DEPENDENT KINASE INHIBITOR SIAMESE

4.1 Introduction

Regulation of cell proliferation and differentiation is necessary for coordinated growth and proper development of organisms. Coordination of cell proliferation and growth is achieved by regulating the passage of the cell cycle through the specific checkpoints in G1 and G2. Transition through these checkpoints is controlled by serine-threonine kinases known as Cyclin Dependent Kinase (CDKs). CDK activity is regulated by a variety of different regulatory proteins for example by binding of cyclin (CDK catalytic partner), CDK activating kinase (CAK), and by CDK kinase inhibitors (CKI) (Mironov et al. 1999; Pines 1999; Boniotti and Gutierrez 2001). CKI proteins were first identified as negative cell cycle regulators in the upstream developmental and environmental growth-signaling pathway of an organism (Wang and Garabedian 2003; Roeder et al. 2010). The animal Kip/Cip family of CKIs, P^{21}, P^{27} and P^{57} were initially identified as tumor suppressers negatively regulating cell proliferation. However, later it was discovered that they are involved in the regulation of transcription, cell migration, and apoptosis (Besson et al. 2008). Animal Kip/Cip proteins are unstructured and has the ability to adopt structures after binding to a protein, which provides them structural flexibility that explains how they can interact with a variety of proteins to regulate different cellular functions (Lacy et al. 2004; Wang et al. 2011).

INTERACTOR OF CYCLIN DEPENDENT KINASES/KIP-RELATED PROTEINS (ICKs/KRPs), named for their distant sequence similarity with mammalian CDK kinase inhibitors protein, and *SIAMESE/SIAMESE RELATED PROTEINS (SIM/SMRs)* are two CDK

inhibitor families in plants (Wang et al. 1997; De Veylder et al. 2001; Churchman et al. 2006). *KRPs* share only 30 amino acid residues of their C-terminal domain with animal counterparts Kip/Cip. Despite this limited similarity between plant and animal kinase inhibitors (CKIs), KRPs inhibit the kinase activity of CYC/CDKs complexes (Lui et al. 2000; Weinl et al. 2005; Nakai et al. 2006), and like animal Kip/Cip proteins, KRPs are negative cell cycle regulators (Wang et al. 1997; Lui et al. 2000). All KRPs are nuclear localized, and only KRP6 and KRP7 contain the consensus CDK phosphorylation (S/TPXK/R) sequence (De Veylder et al. 2001; Zhou et al. 2006; Bird et al. 2007). KRPs spatial expression suggests different functions in plant development (Ormenese et al. 2004)

SIAMESE is the founding member of the *SIAMESE/SIAMESE RELATED PROTEINS (SIM/SMRs)* CDK inhibitor gene family, which are found in all land plant genomes. SIM was initially discovered based on its role in endoreplication. Endoreplication is a modified version of the cell cycle in which DNA replicates without subsequent mitosis and cytokinesis, consequently in each round of replication the amount of genetic material is doubled. Mutation in the SIM gene changes unicellular polyploid trichomes into multicellur trichomes (Churchman et al. 2006). SIM has been implicated in interacting with both CDKA;1 and CDKB1;1 (Churchman et al. 2006; Peres et al. 2007; Van Leene et al. 2010), and it inhibits the kinase activity of CDKs to promote endoreplication in the developing trichomes of *Arabidopsis*. The rice SMR EL2 complements the *sim* phenotype and interacts with D-type cyclins and CDKA;1, in addition, EL2 inhibits CDKA;1 kinase activity in vitro. *SIAMESE-RELATED PROTEIN1* (*SMR1/LGO*), another founding member of the SMR family, was identified during the study of endoreplication in the development of *Arabidopsis's* sepal (Roeder et al. 2010). Mutation in *SIAMESE* and *SMR1/LGO*

genes changes an endoreplicated cell into mitotically dividing cell during the trichome and sepal development, respectively (Walker et al. 2000; Roeder et al. 2010).

SIM/SMRs family members may be involved in mechanisms other than cell cycle. A role for SIM in the Gibberellins (GA) signaling pathway was postulated after observing up-regulation of SIM and other CDK inhibitors by the growth repressing DELLA protein (Achard et al. 2009). SIM is also induced in response to pathogens (Chandran et al. 2010). Rice *SMR EL2* expression increases in response to biotic and abiotic stress (Peres et al. 2007). Reactive oxygen species (ROS) induce the expression of SMR5 and SMR7 in DNA damage responses, resulting in inhibition of mitotic division (Yi et al. 2014). Understanding the regulation of CDK inhibitors KRP, SIM and EL2 induced in response to stress may provide a novel approach to understand the mechanism of the signaling pathway from external stimuli to the molecular level (Churchman et al. 2006; Peres et al. 2007; Rymen et al. 2007; Achard et al. 2009; Yi et al. 2014).

The SIM/SMR protein family is defined by a series of short protein sequence motifs. In this study, we have used site-directed mutants to test the functions of conserved amino acid residues of SIM, and identified a potential phosphorylation site that is essential for function as well two nuclear localization sequences of SIM protein. We have also found that another motif, identified as a CYC/CDK binding site in another study (Peres et al. 2007), is not essential for SIM function.

4.2 Motifs-1 and 2 are essential for SIM function

SIM Motif-1 and -2 are the most conserved protein sequence motifs among all SMRs family members (Chapter-3 results), and were assumed essential for the protein function. To test the necessity of these motifs for the SIM function, I have substituted threonine-34, 35 (from Motif-1), and proline-36 (from Motif-2) to alanines. Over-expression of $SIM^{T34AT35AP36}$ construct

under *GLABRA2 (GL2)* promoter fails to complement the multicellular *sim* trichomes (Figure 4.1C), which supports the hypothesis that this motif is essential for SIM function.

Then, I tested the importance of the proline-rich motif-2 by mutating four prolines (P51-P54) to alanine residues. This Motif-2 mutant fails to complement *sim* when expressed in trichomes (Figure 4.1D). Even though it still interacted with CDKA;1, it does not complement the *sim* phenotype. Because of the special cyclic side chain, proline might be important for protein structural organization and substitution of this amino acid may result in disorganization. To test the stability of proline (Motif-2) mutants, *YFP: SIM*$^{p51\text{-}54\rightarrow AAAA}$ was expressed in developing trichomes under the control of the *GLABRA* (*GL2)* promoter.

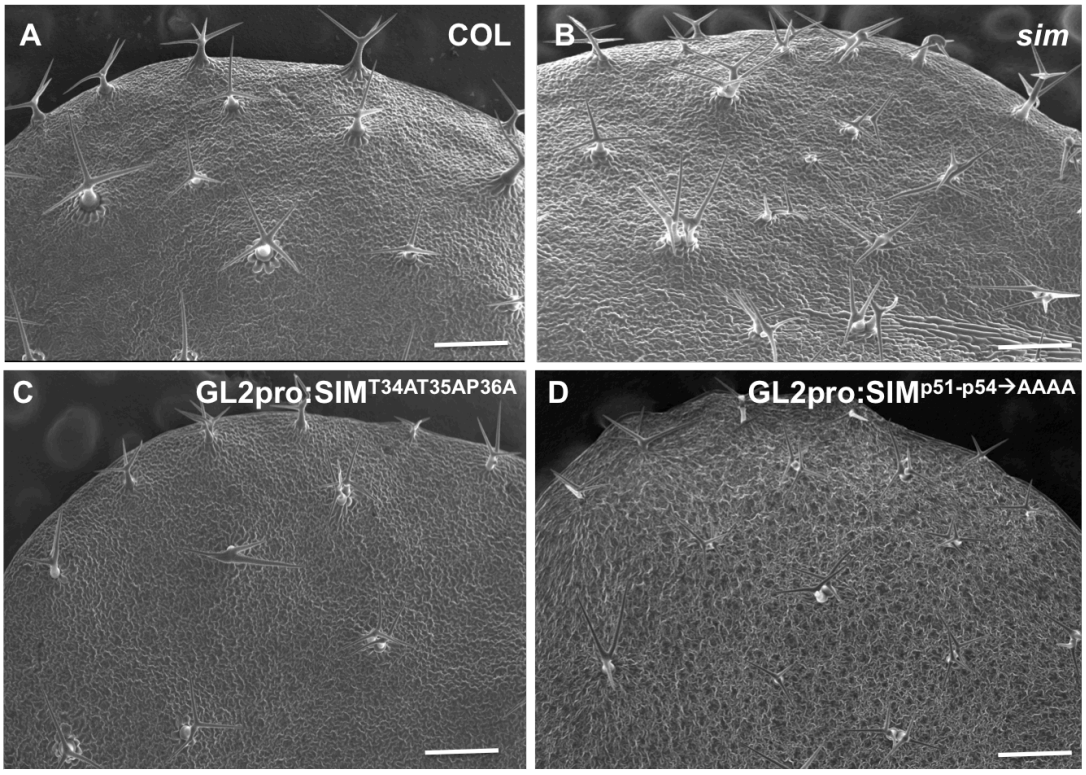


Figure 4.1 SIM Motif-1 and 2 seems essential for protein function. (A) Wild-type Trichome (B) Multicellular *sim* trichomes (C) Over-expression of *SIM*^{T34AT35AP36A} (D) and *SIM*$^{P51\text{-}P54\rightarrow AAAA}$ under GL2 promoter in *sim* background plants. Scale bars are 200 μm.

Table 4.1 All mutants from different motifs tested for their complementation ability, only mutants from Motif-1 and 2 do not complement *sim* phenotype (Y-yes, N-Not).

The *sim* Mutants	Motifs	Complementation
T34T35P36→AAA	1	N
T35A	1	N
P51P52P53P54→AAAA	2	N
R74A	3	Y
C72A	3	Y
L76A	3	Y
R74L76→AA	3	Y
C72R74L76→AAA	3	Y
K73AK75A→AA	3	Y
F96A	4	Y
F95A	4	Y
F95F96→AA	4	Y
E91E93→AA	4	Y
Motif-4 EIERFF	4	Y
S120D	5	Y

Almost 40 independent homozygous transgenic lines were screened for YFP expression in developing trichomes, but YFP fluorescence was never detected. Thus, mutation of this proline-rich motif may destabilize the protein in developing trichomes.

Mutations were constructed in conserved sequences in the remaining motifs (3, 4 and 5) to test their role in SIM protein function, but surprisingly mutation in these motifs was not able to affect the SIM function because all mutants complemented the *sim* trichome phenotype (Table 4.1). Mutation in Motif-1 and 2 seems affect SIM protein function (Table 4.1).

4.3 Threonine-35 (T-35), a potential phosphorylation site in Motif 1, is necessary for SIM function

The SIM protein contains three threonines that are followed by proline, and a serine or threonine followed by a proline (S/T-P), which are the potential target sites for phosphorylation

by CDKs. Therefore, to investigate the potential phosphorylation site in SIM protein, these three threonines were mutated to alanine and the construct was expressed under the *GLABRA2* promoter (*GL2pro*). This tested for their ability to complement the *sim* mutant multicellular trichome phenotype. Substitution of all three threonines to alanines results in a gene that fails to complement the *sim* trichome phenotype (Figure 4.2C), indicating that at least one of these threonines is essential for protein function. Further, mutation of threonines T50 and T63 together does not seem to affect protein function, as this mutant complements the *sim* trichome phenotype (Figure 4.2D).

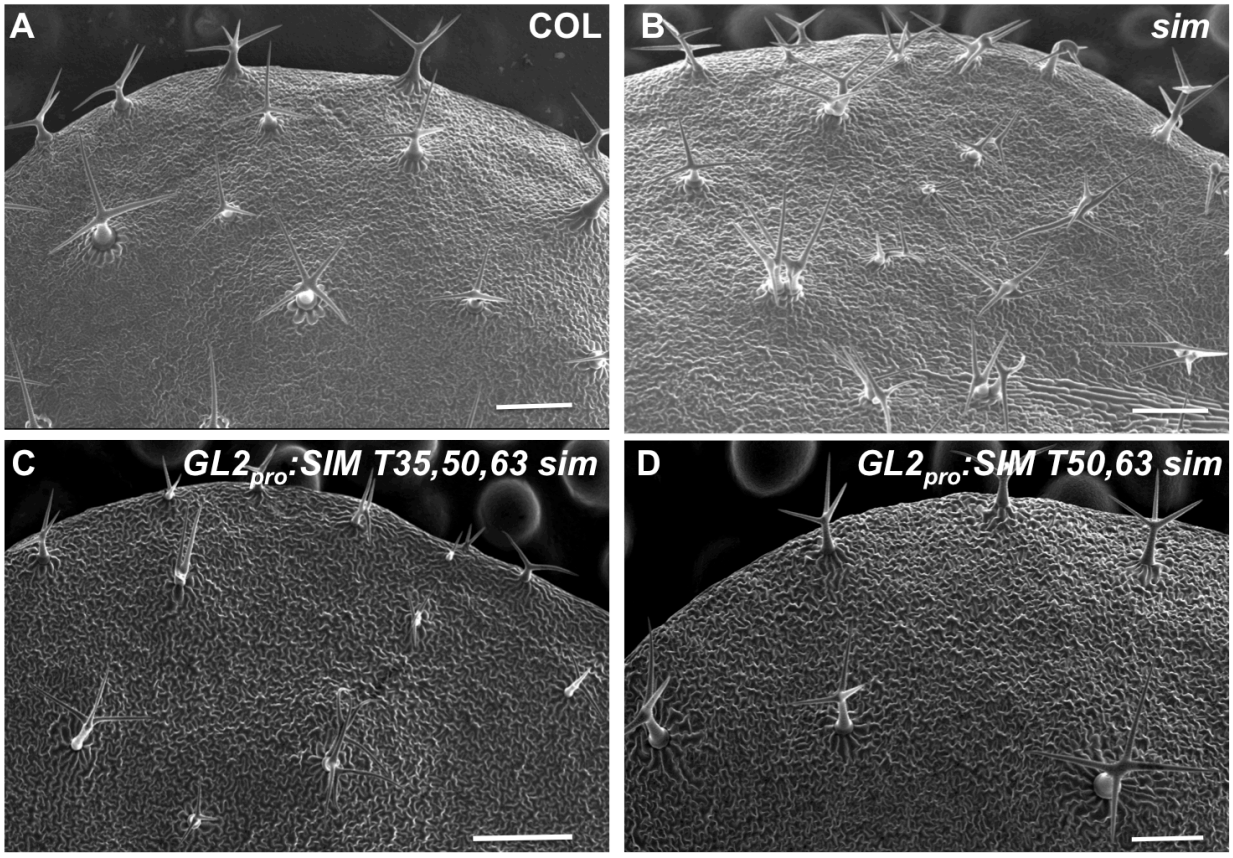


Figure 4.2 SIM-Threonine 50 and 63 are not necessary for SIM function in the *Arabidopsis* trichome (A) Wild-type trichomes (B) multicellular *sim* trichomes (C) SIM in which substitution of T35, T50 and T63 with alanines fails to complement *sim* phenotype, whereas (D) SIM in which substitution of both T50 and T63 to alanines complements *sim* trichome. Taken together, these results suggest that only T35 is necessary for SIM function. Scale Bars are 200 μm.

To examine the role of T35, we substituted this threonine with alanine (T35A), and with the phosphomimic residues aspartate and glutamate. The alanine substitution fails to complement *sim*, confirming that this residue is essential (Fig. 4.3C, Table 4.1). Substitution of T35 with aspartate, on the other hand, is able to complement *sim*, largely restoring the unicellular wild-type trichome phenotype, while the T35E substitution is unable to complement (Fig. 4.3D, E, Table 4.2).

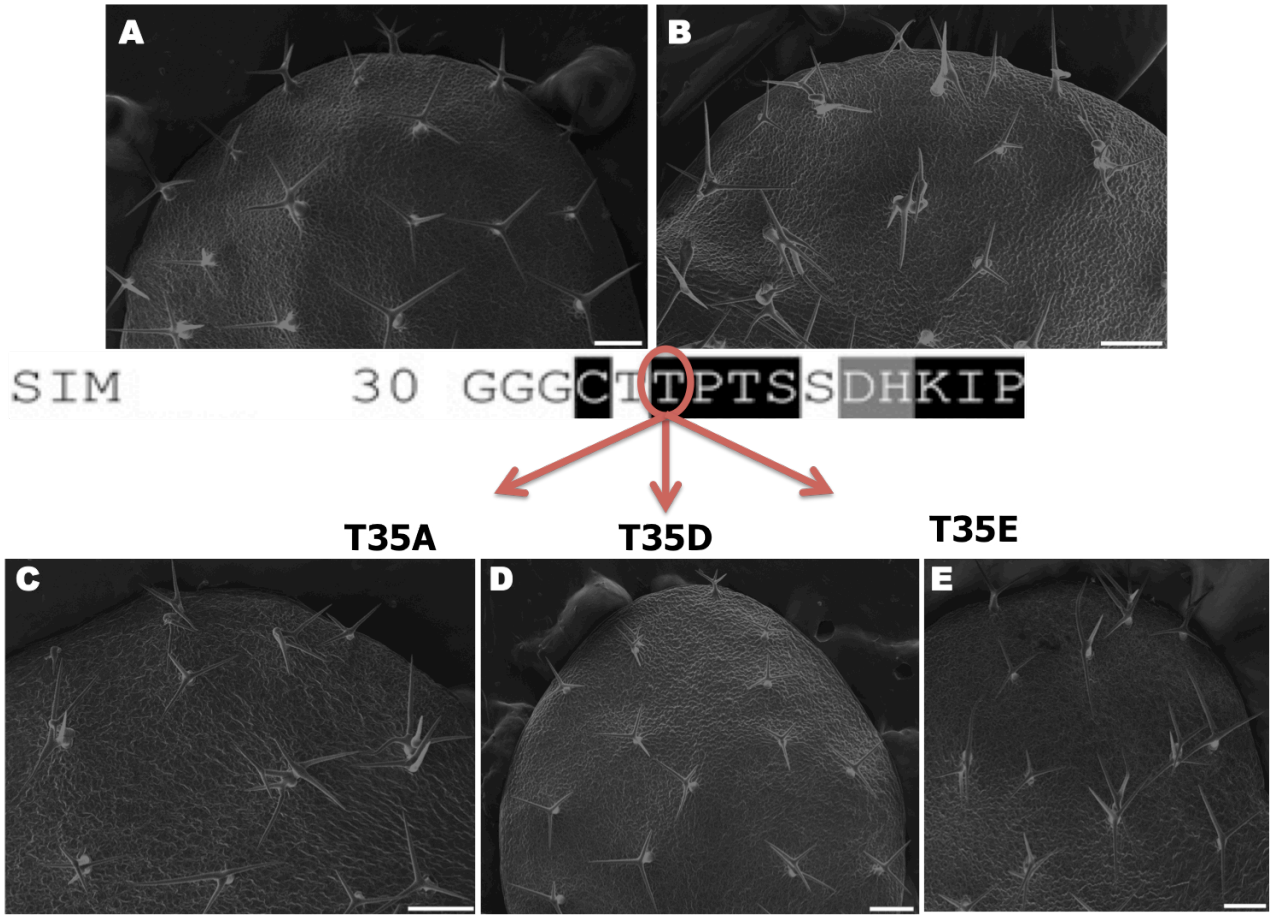


Figure 4.3 Threonine-35 (T-35) is necessary for SIM protein function and a potential CDK phosphorylation site. (A) Wild-type trichome (B) Multicellular *sim* trichome (C) T35A fails to complement the sim phenotype (D) T35D complements *sim* (E) T35E fails to complement *sim,* Scale Bars are 200 μm.

Table-4.2 Number of nuclei in the trichome showing SIM^{T35D} has restored Wild-type trichomes. The number of DAPI-stained trichome nuclei at each trichome initiation site (TIS) were counted for each genotype.

Genotype	Nuclei/TIS	Total no of TIS
Col-0	1.0±0.0	50
Sim	2.5±1.1	50
T35A	2.22±0.8	50
T35D	1.06±.02	50
T35E	1.86±0.9	50

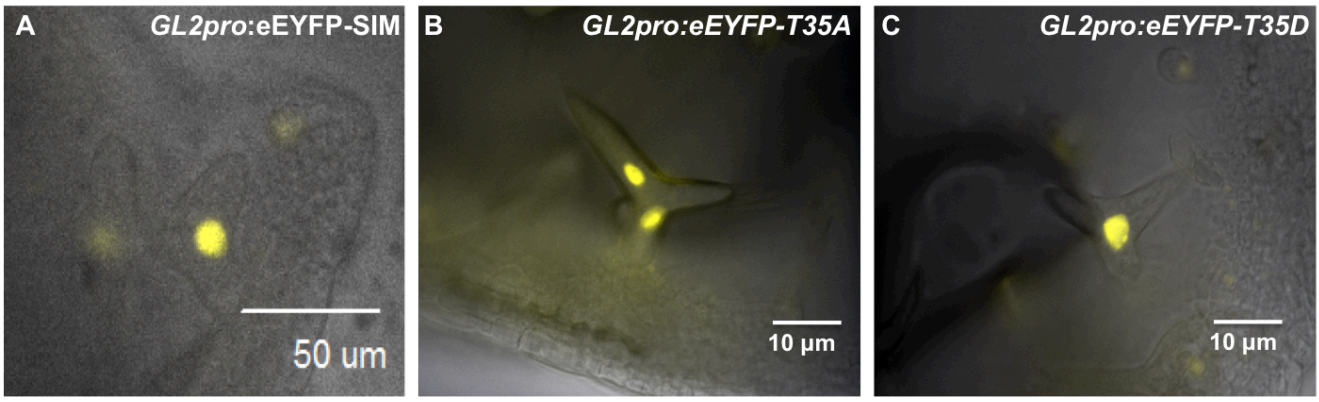


Figure 4.4 SIM, T35A and T35D proteins are stable and nuclear localized in *Arabidopsis* trichomes. Yellow Fluorescence Protein (YFP) tagged at the N-terminal of (A) SIM (B) *SIM-T35A*, and (C) *SIM-T35D* expressed under GL2 promoter.

To confirm that SIM, SIM^{T35A} and SIM^{T35D} lines are expressing and producing stable protein, Enhanced Yellow Fluorescence Protein (eYFP) fusions of SIM and the T35A and T35D mutants were expressed in trichomes under control of the *GLABRA2 (GL2)* promoter, and developing trichomes were examined. All three constructs showed nuclear-localized YFP

fluorescence, indicating that they produced stable and correctly localized protein (Fig 4.4 A, B, and C).

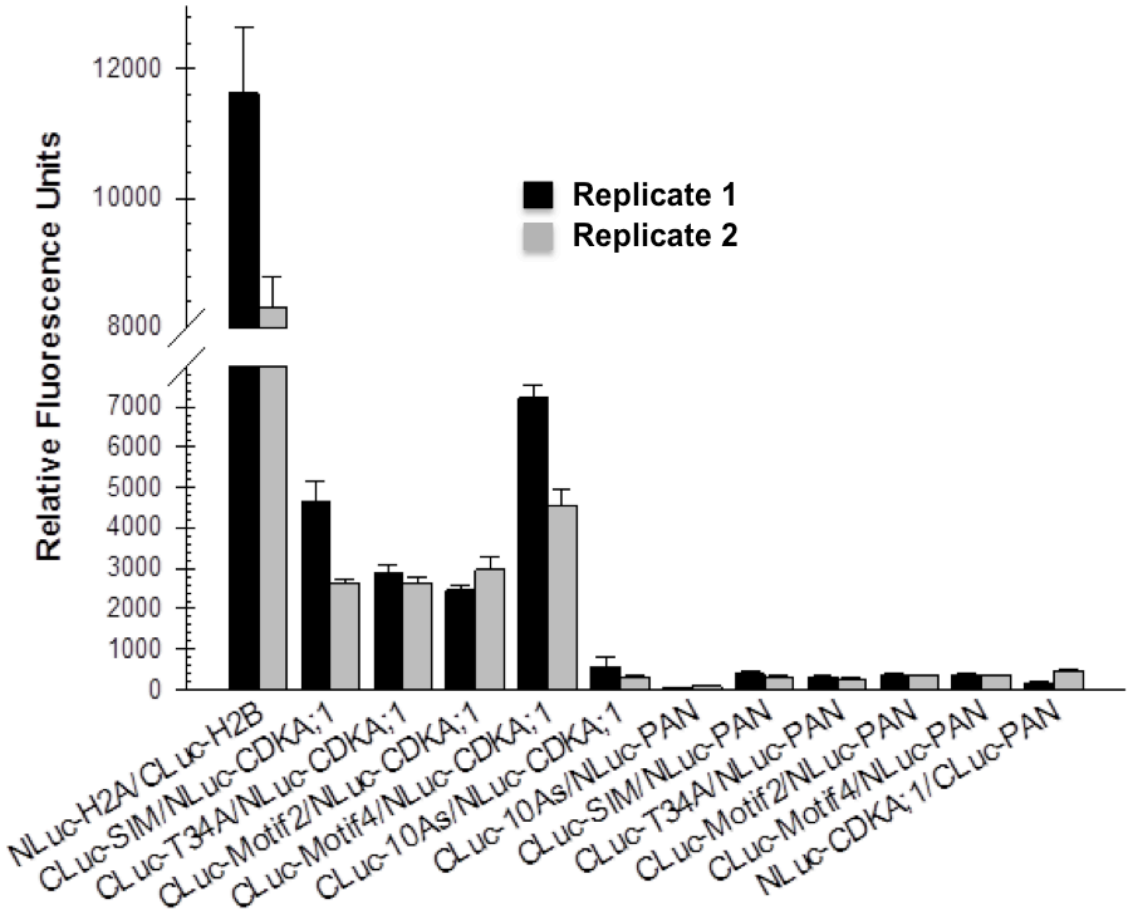


Figure 4.5 Interaction of mutants in SIM Motifs 1, 2 and 4 with CDKA;1 in split-luciferase assay. Interaction of histones H2A and H2B served as a positive control, and interaction with the nuclear-localized transcription factor PERIANTHIA (Yan et al.) served as a negative control. All mutant SIM constructs showed significant interaction above background with CDKA;1, except a mutant replacing motif-1 with alanines.

4.4 Motif-1 appears to be important in SIM interaction with CDKA;1

Split Luciferase Complementation (SLC) assay has shown that SIM interacts with CDKs (Chapter-3). To identify CDKA:1 binding sequence, I have used Motif-1, 2 and 4 mutants in split

luciferase assay to test their ability to interact with CDKA;1. But no mutant was able to abolish the interaction between SIM and CDKA;1, except a mutant where the entire Motif-1 is replaced to alanines (T35 to P44) (Figure 4.5).

I have also tested the longest C-terminal deletion, SIM195, which has only 195 nucleotides out of 384 (or SIMΔ 66-127, which has just 65 amino acid residues), for interaction with CDKA;1 in split luciferase assay. This deletion mutant contains only Motifs-1 and -2 and unexpectedly, interacts with CDKA;1 stronger than positive control histone 2A (H2A) and histone 2B (H2B) proteins (Figure 4.6). This result further opens the questions about stability and necessity of the Motif-1 and -2 for SIM function.

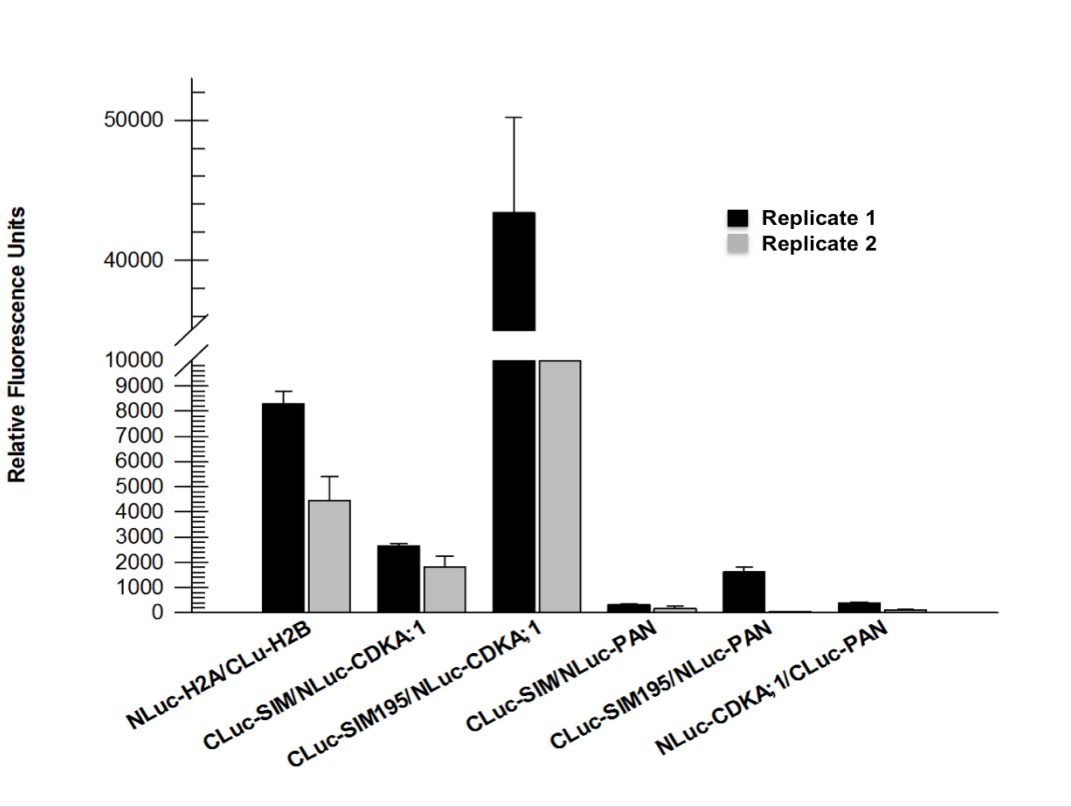


Figure 4.6 SIM deletion (SIM195 or SIMΔ 66-127), where Motifs-3, 4, and 5 are removed at the C-terminus, interacts with CDKA;1 better than positive control. Histone 2A (H2A) and Histone 2B (H2B) transcriptional factor PERIANTHIA are used as positive and negative control, respectively.

4.5 Motif 4, a putative cyclin binding domain, is not required for SIM function.

Motif-4 of SIM (EIERFF) is similar to the KIP-RELATED PROTEIN (KRP) cyclin binding domain and is predicted to be a cyclin binding domain in SIM (Churchman *et al.,* 2006). The rice SIAMESE-RELATED PROTEIN (SMR) EL2 also has a similar domain and has the ability to complement the multicellular *sim* trichome phenotype in *Arabidopsis* (Churchman et al., 2006). Moreover, rice EL2 interacts with CYCD and CDKs, and mutation of the six amino acids of Motif 4 to alanine abolishes interaction of EL2 with CYCDs (Peres *et al*., 2007). In Chapter-3 of this dissertation, we have shown that SIM also interacts with and inhibits kinase activity of CDKs. Therefore, we have assumed that substitution of the amino acid sequence EIERFF to alanine (AAAAAA) would abolish the interaction of SIM with CYCD and CDKs and should lose the ability to complement *sim* trichome phenotype.

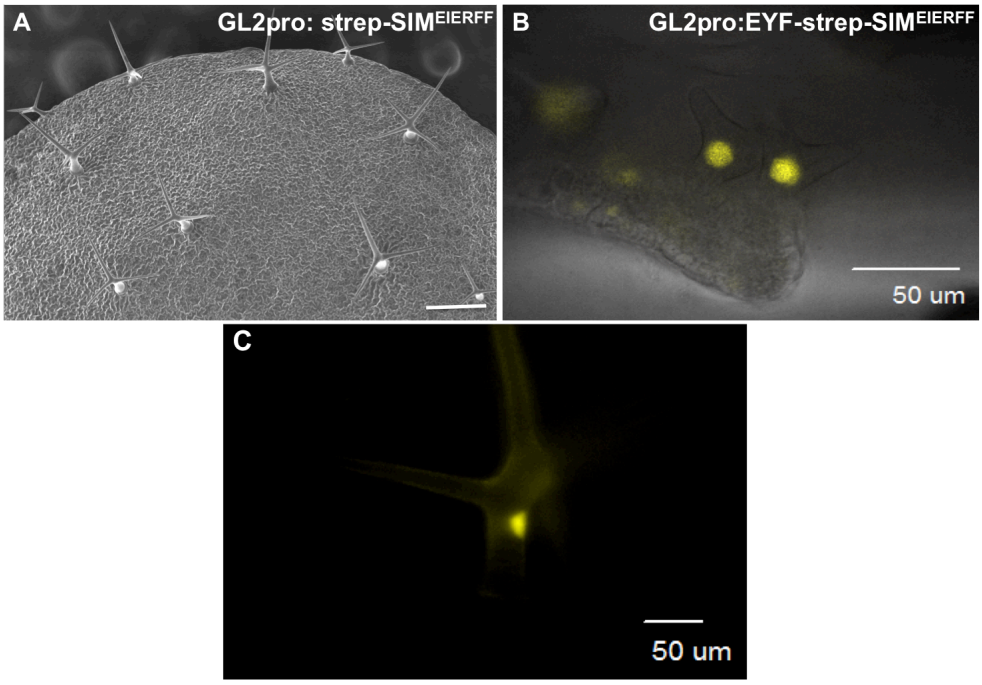


Figure 4.7 SIM Motif-4 (EIRFF) does not seem essential (A) SIM lacking Motif-4 complements *sim* phenotype when expressed under *GL2* promoter (B) GL2: eYFP-Strep-SIM is stable and expressed in the developing trichome nucleus (C) Yellow Fluorescence in mature Trichome. Scale Bar for panel (A) is 200µm.

Surprisingly, even after substitution of all six amino acids of Motif-4 (EIERFF) to a stretch of alanines (AAAAAA), the mutant gene is still functional and restores the wild-type trichome phenotype (Figure 4.7A). Enhanced Yellow Fluorescence (eYFP) protein tagged *GL2pro: eYFP-strep-SIM*$^{EIERFF\ to\ AAAAAA}$ was expressed in the *Arabidopsis sim* trichomes and nicely restored SIM function (Figure 4.7B,C), in addition, yellow fluorescence observed in trichomes illustrates that the protein is stable and localized in the nucleus. SIM Motif-4 mutant interacts with CDKA;1 (Fig 4.5). All conserved amino acids in motif-4 were mutated in different combinations and tested for their effect of the SIM function; all mutant complemented the *sim* trichomes (Table 4.1).

4.6 SIM has two nuclear localization sequences (NLS)

SIM/SMRs family members are exclusively nuclear localized proteins (Churchman et al., 2006). Motifs 3 and 5 are primarily short stretches of basic amino acids, resembling known nuclear localization sequences. Therefore, to identify the Nuclear Localization sequences 'NLS', we have created systematic deletions, and substituted specific (potential 'NLS') amino acids of SIM protein (Figure 4.8). Green Fluorescence Protein (GFP) was fused to the N-terminus of SIM and various deletion and substitution mutants, and these constructs were transiently expressed in the tobacco leaves. A C-terminal deletion removing Motif-5, which may contain a putative NLS sequence, results in GFP:SIM localization primarily in the nucleus, with a small amount of protein in the cytoplasm (Figure 4.8C). After deletion of motifs-4 and -5, GFP:SIM showed clearly increased cytoplasmic expression (Figure 4.8D), while a deletion removing Motifs-3, -4 and -5 resulted in distribution of GFP:SIM relatively equally between the nucleus and the cytoplasm (Figure 4.8E). A nuclear-localized wild-type GFP fusion of SIM (GFP:SIM) and

cytoplasmically localized Beta Carbonic Anhydrase-2 (GFP-βCA2) were used as controls (Figure 4.8 A, B).

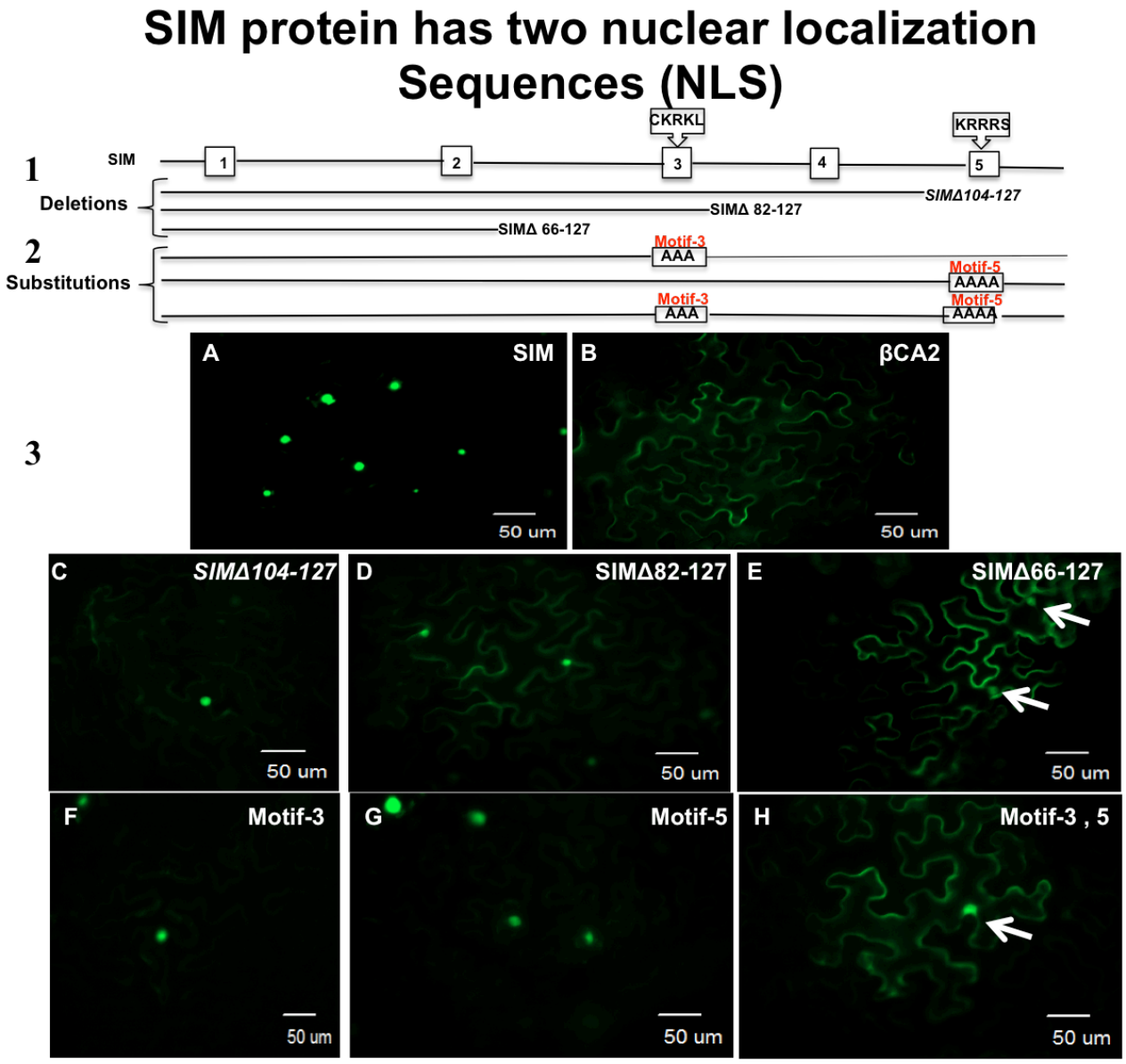


Figure 4.8 SIM has two Nuclear Localization Sequences. Subcellular localization of GFP:SIM fusion proteins was tested by transient expression in tobacco leaves. (1 and 2) Schematic representation of the SIM deletions and substitution mutations in Motif-3 and 5, respectively. (3) Localization of SIM and their mutants (A) Nuclear localized SIM (B) Cytoplasmic localized Beta Carbonic Anhydrase-2 (βCA2) (C) C-terminal deletion removing motif-5 (D) C-terminal deletion removing Motif-4 and Motif-5 (E) C-terminal deletion removing Motifs-3, -4 and -5 deleted (F) Substitution of basic residues of Motif-3 (KRK) to alanines, and (G) Substitution of Motif-5 (KRRS) with alanines. (H) Substitution of both Motif-3 and 5 sequences to alanines.

Deletion results have clearly demonstrated that NLS sequences are present in the C-terminus of SIM in or close to Motif-3 and Motif-5; therefore, to test the exact amino acids involved in nuclear localization, we mutated the underlined amino acids of in Motif-3 (CKRKL), and Motif-5 (KRRRS) to alanines. These sequences individually are not able to change the localization of GFP fusions substantially (Figure 4.8F,G), but when both Motifs were mutated, GFP:SIM was found equally in the cytoplasm and the nucleus (Figure 4.8H). Hence, motif-3 and motif-5 act as duplicate NLS sequences.

We also tested the ability of these mutant genes to supply *SIM* function by testing their ability to complement the *sim* mutant trichome phenotype in *Arabidopsis*. The deletions removing motif-5, or both motifs-4 and -5, were able to complement *sim* (Figure 4.9A,B). However, the deletion removing motifs-3, -4, and -5 was not able to complement *sim*. Preliminary results indicate the substitution mutants in either motif-3 or motif-5 independently can complement *sim*, but when both motifs are substituted by alanines, the gene is nonfunctional(data not shown because plants were not ready to take SEM images). Thus it appears that targeted nuclear localization of *sim* is required for function.

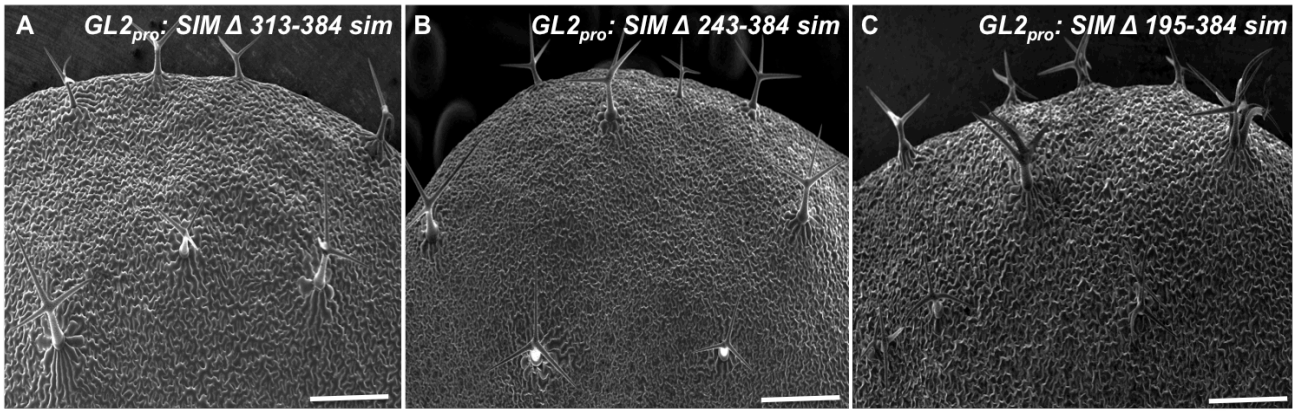


Figure 4.9 SIM deletion, where motif-3, 4 and 5 is deleted, unable to complement multicellular trichome (A) C-terminal deletion removing Motif-5 complements the *sim* trichome phenotype (B) A C-terminal deletion removing both Motif-4 and Motif-5 also complements *sim* (C) A C-terminal deletion removing Motifs-3, -4 and -5 does not complement *sim*.

4.7 Discussion

In chapter-3 of this dissertation, we have shown that SIM interacts with and inhibits the activity of several CYC/CDK complexes. To find the amino acids involved in the interaction with CDKs, I used sited-directed mutagenesis, and tested functionality of the mutants by genetic complementation in transgenic *Arabidopsis* plants and Split Luciferase protein-protein interaction assays in the *Arabidopsis* protoplast. Mutations affecting motif-1 or motif-2, the most highly conserved motifs in the *SMR* family, failed to complement *sim* mutants, indicating that these motifs are essential for in vivo function (Figure. 4.1C, 4.3D and Table 4.1). A mutation substituting all ten amino acids of motif-1 with alanines disrupts the interaction between SIM and CDKA;1, suggesting that motif-1 is a CDK interaction domain (Figure 4.6). We obtained YFP:tagged lines showing fluorescence in developing trichomes for non-complementing substitutions in T35 of motif-1, indicating that though these motif-1 mutants are nonfunctional, they still produced nuclear-localized protein. The observation that T35, the most evolutionarily conserved residue in the *SMR* family, is located in a potential CDK phosphorylation site, and that the phosphomimic substitution T35D is functional, suggests that phosphorylation of T35 may play a key role in regulating SIM.

In contrast, an alanine substitution mutant in the proline-rich motif-2 failed to complement *sim*, and we were unable to detect fluorescence when this mutant was fused to YFP, suggesting that the protein was unstable or not expressed.

Motif-4 has been implicated as a potential cyclin-binding motif, based on its role in the related KRP family of CDK inhibitors and on its importance in interaction of EL2, a rice SMR, with a D-type cyclin (Churchman 2006, Peres 2007). Because this Motif is implicated in cyclin binding and not CDK binding, it is not surprising that it does not affect interaction with CDKA;1

(Figure. 4.5). More surprising is the observation that this motif is not essential for *SIM* function in trichome development (Figure. 4.7). This Motif shows significant conservation in the *SMR* family (Chapter 3), and it may be required for interaction with cyclins during function of SIM in other tissues, or perhaps expression from the *GL2* promoter, known to be a strong promoter, increased SIM protein concentration to a level where cyclin interaction is not needed.

Surprisingly mutation in any amino acid of Motif-3 and Motif-4 does not affect SIM protein function. Mutants from these Motifs complemented *sim* phenotype, and were not able to abolish the interaction with CDKs (Figure 4.5, Table 4.1). Then, systematically we mutated all conserved amino-acid residues, but all mutants failed to abolish the SIM interaction with CDKA;1 except in Motif-1 when all 10 amino acids together were substituted to alanines (Figure 4.5). How the substitution of these 10 amino acid affects the protein function needs further study.

SIM Motif-2 (PPPPPQKPRPP) is proline rich, so we tested this motif role in SIM and CDK interaction. Even after substitution of P51-P54 to alanines, $SIM^{P51\text{-}54\rightarrow AAAA}$ still interacts with CDKA;1, but this mutation does not complement the *sim* phenotype. Therefore, Motif-2 may be required for in the stability or structure of protein.

By making deletions and substituting potential Nuclear Localization Sequences "NLS" to alanines, two nuclear localization sequences of SIM have been identified (Figure 4.8). When the deletion and substitution mutants were transformed in the *sim* mutant background plants, the complementation results were consistent with localization results (Figure 4.9). If the protein is not located in nucleus, it does not complement the *sim* mutant trichomes. However, the role of Motif-3, 4, and 5 in SIM activity is yet to be determined.

CHAPTER 5
IDENTIFICATION OF AMINO ACIDS INVOLVE IN THE SIAMESE (SIM) PROTEIN STABILITY

5.1 Introduction

Protein degradation is essential to regulate the level of protein in different cellular processes including the cell cycle, gene expression, growth and metabolism of eukaryotic cells. Moreover, the irreversibility of the degraded protein and protein half-life are also critical processes to maintain the homeostasis in eukaryotic cells. To maintain the level of a protein, the autophagy-lysosome (a self-digestion process of a cell), and ubiquitin-proteosome pathways are well-known protein degradation pathways in eukaryotic cells. The autophagy-lysosome protein degradation system targets extracellular, cell surface and some cytosolic proteins. Proteins are engulfed and degraded by lysosomes, but some cytosolic proteins are degraded by lysosome via autophagy vacuoles (Mizushima 2007; Levine and Kroemer 2008). Beside ubiquitin-proteosome and lysosome, serine, aspartate, and cysteine proteases are also involved in the protein degradation.

Ubiquitin-proteasome pathway is essential in eukaryotes. Ubiquitin, a highly conserved 76 amino acid polypeptide among eukaryotes, is covalently attached to a target protein, tagging it to be degraded in an ATP-dependent reaction cascade. In this cascade, ubiquitin-activating enzyme (E1) activates ubiquitin by making a thio-ester bond between free C-terminal carboxyl group of ubiquitin and a Cys residue of E1 enzyme. In the next step, this activated ubiquitin is transferred to the active-site cysteine of an ubiquitin-conjugating enzyme again forming a thio-ester bond. Finally, with the help of an ubiquitin-protein ligase (E3), ubiquitin is transferred to the substrate and makes an isopeptide linkage to an ε- amino group of substrate internal lysine. The mechanism to transfer ubiquitin from E2 to E3 varies depending on the involvement of

different E3 ligase domains (HECT and RING). E3 ligases have been divided on the basis of the presence of HECT (Homology to Human E6-Associated Protein C-Terminus) and a RING (Really Interesting New Gene)/U-box domain. HECT E3s form a covalent bond between E2 and substrate, whereas RING/U-box E3s ligases directly transfer ubiquitin from E2 to substrate and act as an adaptor between them. Ubiquitin E3 ligases are the most diversified enzymes in the ubiquitination cascade; more than one thousand E3 ligases have been predicted in *Arabidopsis* (Mazzucotelli et al. 2006). In eukaryotes, multi-subunits E3 ligases are SCF (SKP1-CULLIN-F-box), CUL3-BTB/POZ, CUL3 (CULLIN3), CUL4-DDB1 (UV-Damaged DNA-Binding Protein1) and APC/C (Anaphase Promoting Complex/Cyclosome).

Ubiquitin E3 ligases that play central roles in the eukaryotic cell cycle are Anaphase Promoting Complex/Cyclosome (APC/C), SCF a class of ligase, and Cullin-RING ubiquitin ligases (CRLs)**.** SCF is involved in G1 to S transition phase and is thought to target cyclin dependent kinase inhibitors (CKIs) (Vodermaier 2004; Nakayama and Nakayama 2006). F-box proteins in SCF E3 ligases act as adapters and determine the substrate specificity and functional variety of the SCF (Bai et al. 1996; Kipreos and Pagano 2000). APC, a multisubunit ubiquitin protein ligase (E3) complex, degrades the proteins involved in the metaphase to anaphase transition and targets mitotic cyclins destruction box (D-box) for ubiquitination. The D-box sequence is "RQVLGDIGN" at the N-terminal of cyclins (CYCA and B) is recognized by APC as substrate. The *CELL CYCLE SWTICH PROTEIN52* (*CCS52* known as Cdh1/FZR in animals) and Cell division cycle (Cdc20) are two important activators that determine the substrate specificity of APC E3 ligase complex. Therefore, APC E3 ligase ubiquitination reaction includes E1, E2, E3 ligase and either Cdc20 or CCS51A activator. Cell division cycle (Cdc20) containing (APC^{Cdc20}) is involved in early and mid mitotic phase and degrades mitotic A and B-type cyclins,

securins, and promotes chromosome separation. $APC^{CDH1/CCS51}$ specifies the degradation of late mitotic proteins and prevents reaccumulation of mitotic cyclins during G1 phase. There are many CULLLIN RING ubiquitin ligases (CRLs) in plants and all CRLs are thought to share RING finger protein RBX1 (Lechner et al. 2002). The poly-ubiquitinalated protein is then recognized and degraded by the 26 proteasome (Smalle and Vierstra 2004). The 26 proteosome is a multicatalytic protease complex consisting of a cylindrical 20S core protease and both ends of this cylindrical structure are covered by 19S regulatory particles (Desterro et al. 2000; Groll and Huber 2003).

Endoreplication is a cell variant in which cells pass through multiple rounds of DNA replication without mitosis, resulting in polyploid genomes. Endoreplication occurs in mature or differentiated cells (Castellano Mdel et al. 2004; Garcia-Higuera et al. 2008; Fox and Duronio 2013), and is crucial in early developmental stages (Garcia-Higuera et al. 2008; Lee et al. 2009b). High DNA content in the endoreplicated cell is correlated with cell volume and high metabolic rate, so endoreplication is more advantageous where cell surface is necessary with limited sources of energy (Kondorosi et al. 2000; Edgar and Orr-Weaver 2001; Inze and De Veylder 2006). Endoreplication plays a decisive role in cell fate and tissue morphology in plant development (Walker et al. 2000; Bramsiepe et al. 2010; De Veylder et al. 2011).

Small ubiquitin-related modifiers (SUMOs) are post translation modifiers, and they are attached to lysine (K) in an ATP-dependent enzymes cascade (Johnson 2004). Modification of the protein by SUMOylation could change the subcellular localization and it has been implicated in many cellular processes, including cell cycle regulation, transcription, signal transduction, and DNA replication and repair (Geiss-Friedlander and Melchior 2007; Ishida et al. 2009; Kanakousaki and Gibson 2012). SUMO E3 ligases, HIGH PLOIDY 2 (HPY2), repressed the

onset of endoreplication and modulated the cell cycle progress in *Arabidopsis* meristem development (Ishida et al. 2009).

SIAMESE is a negative cell cycle regulator, and conversely promotes endoreplication in the *Arabidopsis* trichome development (Walker et al. 2000; Churchman et al. 2006). Mutation in the SIM gene changes unicellular wild-type trichomes into multicellular trichomes (Walker et al. 2000). As hypothesized previously, and demonstrated in chapter-3 of this dissertation, SIM promotes the onset of endoreplication by inhibiting the activity of one or more CDK complexes. SIM protein has eight lysine residues and Lys residues are the sites where ubiquitin is attached to mark a protein to be degraded. For other plant CDK inhibitor family members, KRP1, KRP2 (KIP-RELATED PROTEINS), degradation is mediated by ubiquitin-proteosome pathway (Jakoby and Schnittger 2004; Verkest et al. 2005b; Ren et al. 2008) and ubiquitin-mediated protein degradation is a common means of cell cycle regulation, enforcing unidirectional progression through the cell cycle. It seems very possible that SIM function will be regulated by ubiquitin-mediated proteolysis as well. For this reason, I mutated lysine amino acids of SIM to arginines, which can not be ubiquitinated, and have tested their effect on the functionality of the protein.

5.2 Lysine-42 (K-42) is essential for the SIM protein function

In protein degradation via the ubiquitin-mediated proteosome pathways, ubiquitin chains are covalently ligated to lysine residues and tag proteins to be degraded by 26S proteasomes. The SIAMESE protein contains eight lysine amino acid residues, so it is tempting to test these amino acids role in SIM protein stability. We postulated that mutation in one or more lysine residues will make SIM protein more stable. Overexpression of the mutated protein in *Arabidopsis* trichomes may produce complete cell cycle arrest phenotype seen upon over-expression of the

KINASE-RELATED PROTEINS (KRPs), the other known CDK inhibitor family in plants. Overexpression of ICK1/*KRP1* from the *GL2* promoter inhibits trichome development by inhibiting both the G1/S and G2/M cell cycle transitions, ultimately resulting in cell death (Schnittger et al. 2003) in contrast to wild-type-SIM, which blocks the G2/M transition but allows S phase to proceed.

All eight lysine amino residues were mutated to arginines (all K→R) and over-expressed under trichome specific *GL2* promoter in *sim* and COL plants. Surprisingly, this mutant complemented the *sim* phenotype as well as the wild-type gene (Figure 4.1D), and did not lead to any changes in wild-type trichomes phenotype. We then mutated SIM lysine 42 to arginine (K42R) due to its location within the essential conserved Motif-1 sequence, and expressed the mutant under *GL2* promoter. In an unexpexted contrast to the results from the all K to R mutation, the *SIM-K42R* mutation failed to complement the *sim* phenotype (Figure 4.1C), so K42 appears to be essential for SIM.

Motif-3 of SIM contains two lysines, K73 and K75. Motif-3 was initially thought to be a potential cyclin interaction motif, and we were expecting that mutation in motif-3 should abolish SIM interaction ability with CYC/CDK complex. However, results from this dissertation chapter-4 have shown the role of K73K75 in nuclear localization. We had constructed a mutation converting both of these lysines to alanines (K73AK75A), and this construct complemented *sim,* indicating that these lysines are not essential for SIM function (Figure 5.3).

5.3 Wild-type SIM is degraded by a proteosome-mediated pathway

SIM and *SIM-K73AK75A* were tested by our collaborators Genvieve Lamy and Pascal Genschik (University Strasburg, France) for their roles in the protein stability. GFP-SIM

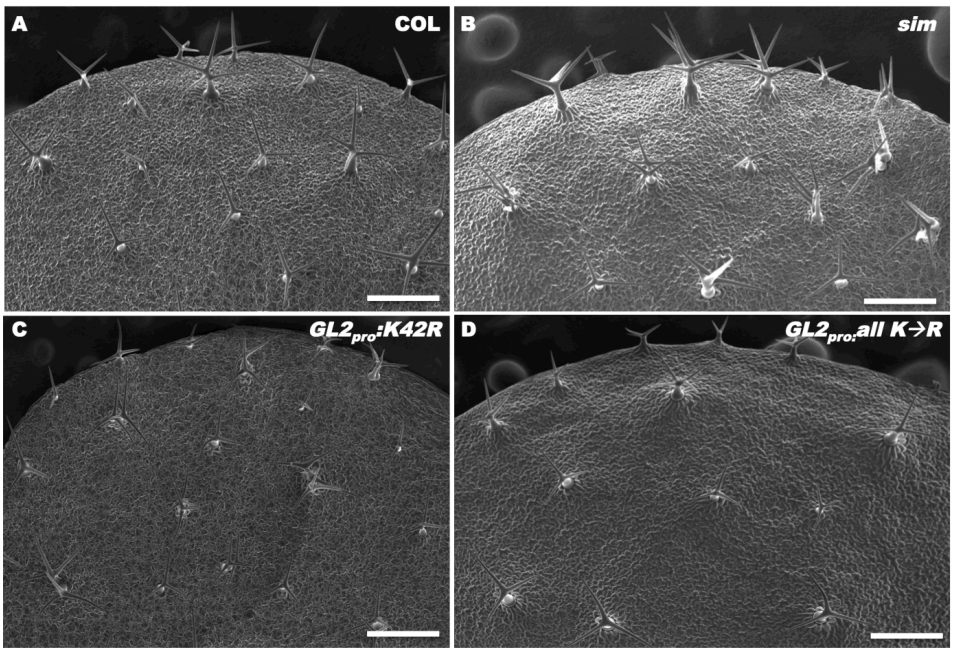


Figure 5.1 Lysine-42 is necessary for the SIAMESE Function in *Arabidopsis* Trichome (A) Wild-Type Trichome on Arabidopsis Leaf (B) *sim* trichomes (C) *proGL2: SIM* K42R in *sim* background plants (D) *proGL2: SIM K→R* in sim background plants. Bar is the same in all panel scale bar is 200 um.

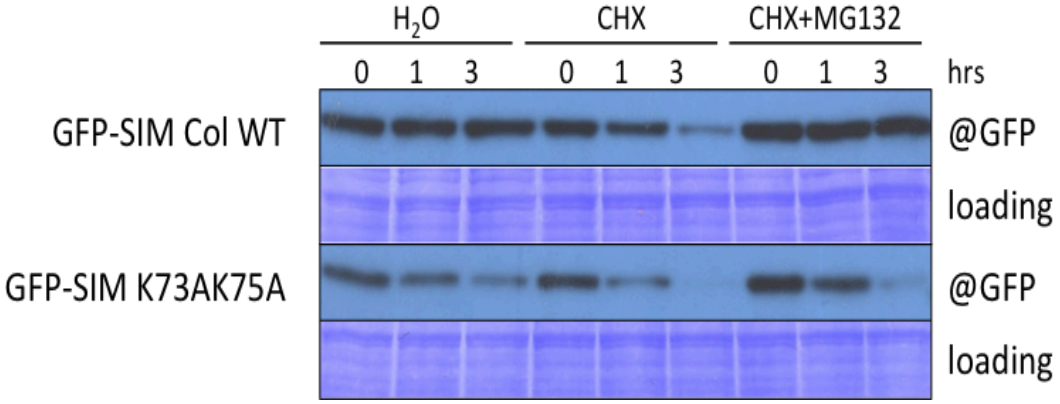


Figure 5.2 SIM is degraded by a proteosome-mediated pathway,and *SIM-K73AK75A* seems degraded by an independent pathway. Green Fluorescen protein tagged (GFP) SIM and SIM-K73AK75A were detected using anti-GFP antibody (Figure is the work of Genvi`eve Lamy and Pascal Genschik (University of Strasburg, France). Protein crude extract is loading control.

and *GFP-K73AK75A* were transiently expressed under 35S promoter in the tobacco leaves and were detected using anti-GFP antibody for their half-life (Figure 5.2). Tobacco leaves disks were incubated in buffer (H_2O), cyclohexamide (CHX) to inhibit protein synthesis, and cyclohexamide plus proteosome inhibitor MG132, and samples were taken to blotting as indicated in figure 5.2.

Clearly wild-type SIM was degraded by proteosomes pathway because after incubation with the proteosome inhibitor MG132, the protein was stabilized (Figure 5.2, upper most lane). In contrast, the *K73AK75A* was less stable, and was not stabilized by MG132, indicating that it is degraded by a proteosome-independent pathway (lower lane figure 5.2). Cyclohexamide decreases protein concentration after one hours in both SIM and SIM-K73AK75A lanes (Figure 5.2). The SIM-K73AK75A under GL2 promoter complemented the *sim* phenotype, but we have observed that some transgenic lines show large epidermal cell and abortive trichome (Figure 5.3). From aborted trichomes, the DNA content was measured and compared with the wild (COL) and *sim* trichomes, but no significant difference was observed among them (Figure 5.4). We also detected the large epidermal cells and abortive trichome in some lines transformed with wild-type SIM, and it is likely to be an artifact due to silencing of endogenous GL2 promoter (Figure 5.3B).

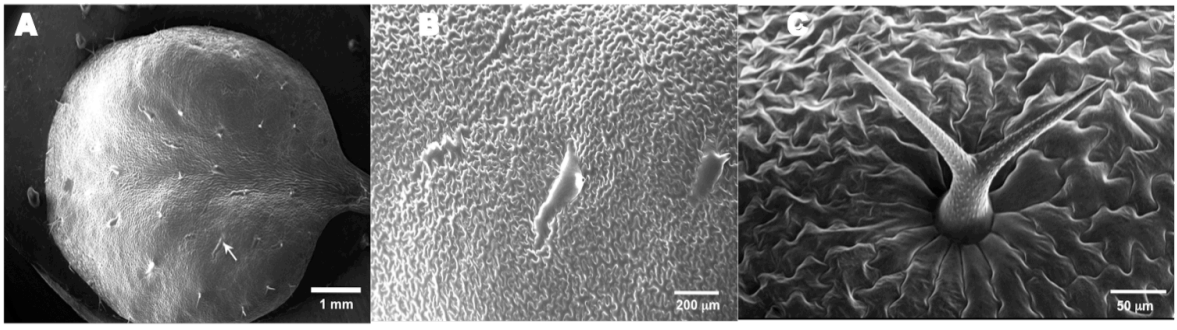


Figure 5.3 *GL2pro: SIM K73AK75A* in *sim* plants inhibits *Arabidopsis* trichome growth (A) 12-days leaf showing large epidermal cells and aborted trichome (B) Large epidermal cell (C) Aborted Trichome.

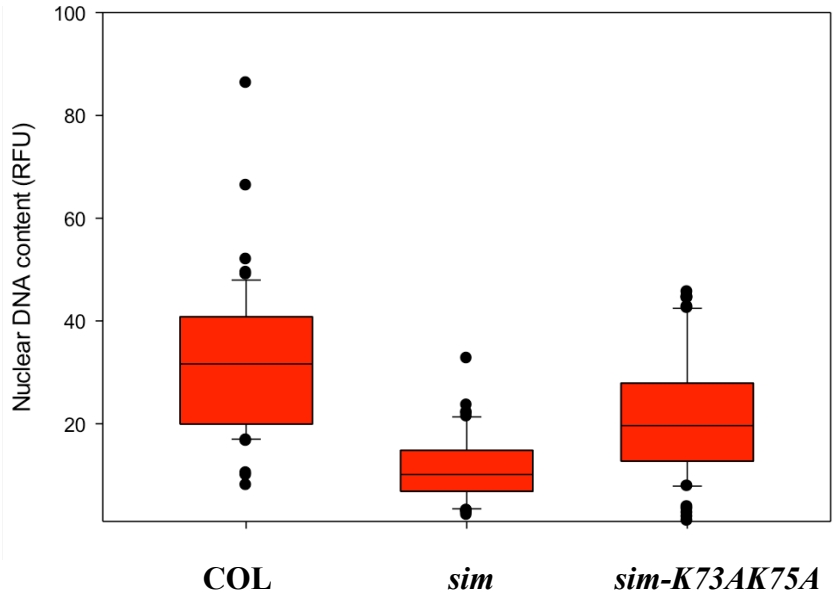


Figure 5.4 Nuclear DNA content of COL, *sim* and *sim*-K73AK75A

5.4 Discussion

If protein is degraded by ubiquitination-mediated pathway, a mutation in target lysines should make the protein more stable. Therefore, mutating SIM lysines in different combination we were attempting to identify the lysines responsible for protein degradation. Surprisingly, mutation of all eight lysine to arginine did not affect protein function at all. However, K42R failed to complement *sim* phenotype and seems important for the protein function. Substitution of all K→R may have counter effect on the K42→R mutation, so compensate K42R mutation. Stability assay indicates that mutation in K73K75 does not make protein stable. K42R kills SIM function, so there might be a possibility that other lysines in any other combination may be responsible for the protein degradation or give clue about their roles in SIM protein degradation mechanism.

One possibility may be that Lysine-42 is involved in the SUMOylation of SIM. SUMOylation tags the protein in the same way as ubiquitination, however the outcomes are

different. Poly-ubiquitination leads to degradation of the protein while SUMOylation modifies the targeted proteins. SUMO (Small Ubiquitin-Like Modifiers) E3 ligase HIGH PLOIDY2 (*HPY2)* is thought in suppressing the endoreplication (Ishida et al. 2009). SIM/SMRs family members, SIM, SMR1, SMR2 and SMR3, have conserved potential SUMOylation site D/E-H-K-I in motif-1 (Matic et al. 2010).

SIM-K73AK75A mutant is expressed under GL2 promoter, so aborted trichome and large epidermal cells might be the result of silencing of endogenous GL2 promoter. Therefore, to test the cause behind the aborted trichome and large epidermal cells phenotype, SIM-K73AK75A has been inserted in TRY_{PRO}*: pEGAD* destination vector, and has been transformed in the *sim Arabidopsis* plants. Mutation in *gl2* produced aborted trichomes on the *Arabidopsis* leaves, so GL2 plays a crucial role in the morphological development of the *Arabidopsis* trichome (Rerie et al. 1994). Mutation in *TRIPTYCHON (TRY)* gene (*try)* leads to formation of trichome clusters and increased endoreplication (Hulskamp et al. 1994; Hulskamp 2004). Both TRY and GL2 are involved and expressed in early trichome development, and their functional loss has opposite effects on trichome development.

CHAPTER 6
CONCLUSIONS

SIM acts as a negative cell cycle regulator in the developing trichome of *Arabidopsis* by inhibiting the kinase activity of CDK kinases, and it is a founding member of the SIM/SMRs gene family. *SIM* mutant gene changes endoreplicated unicellular trichomes into multicellular trichomes (Churchman et al. 2006; Peres et al. 2007). All SIM/SMRs family members share conserved motifs and are assumed to have the same molecular mechanism to perform different functions (In this dissertation chapter-3). Some SIM motifs are shared with another plant CDK inhibitor family known as *KIP RELATED PROTEINs* (*KRPs*) (Churchman et al. 2006). On the basis of *SIM/SMR* family members interaction with different CYC/CDKs complexes, SMRs have been divided into two groups (Van Leene et al. 2010). However, in this study, we have shown that almost all *SMRs* and even *Physcomitrella patens* (PpSMR) complement the *sim* phenotype, indicating that SIM/SMRs family members are functionally conserved in all land plants. On the basis of the study in chapter-3, we have concluded that SIM and even the phylogenetically most diverse *Physcomitrella patens SMR* (PpSMR), in vitro, inhibit the kinase activity of CDKA;1. Additionally, rice SMR EL2 also interacts with CDKA;1 and inhibits CDKA;1 kinase activity (Peres et al. 2007). SIM and SMR1/LGO inhibit mitotic cell division to cause onset of endoreplication in the development of *Arabidopsis* trichome and sepals, respectively (Churchman et al. 2006; Roeder et al. 2010). SMR2 knock-out does not show any phenotypic changes in leaves, but the sizes of leaves are increased, while DNA content remains the same indicating SMR2 role in the cell division and controlling the size and growth of the *Arabidopsis* leaves. Furthermore, almost all *Arabidopsis SMRs* complement *sim* phenotypes in *Arabidopsis* trichomes, which leads to the assumption that all SMR members functionally are same.

Both in vitro and in vivo evidence have established SIM as a CDK kinase inhibitor. In vitro, SIM inhibits the kinase activity of both CDKA;1 and CDKBs, and in vivo, the *cycd3,1-3* mutant complemented *sim* phenotype and restored *Arabidopsis* unicellular wild-type trichome. Moreover, functional loss of both CDKBs (CDKB1;1 and CDKB1;2) in *sim* mutants restored the wild-type trichome phenotype. SIM seems to inhibit CDKA;1 and CDKBs to maintain their low kinase activity to promote endoreplication in the *Arabidopsis* developing trichome. CDKA;1 and CDKBs control the transition in cell cycle from G1 to S and G2 to M phase, respectively (Nowack et al. *2010*; Nowack et al. 2012). CDKA;1 increases CDKB1;1 kinase activity by phosphorylating and inactivating *RETINOBLASTOMA RELATED PROTEIN* (*RBR*). CDKBs are the target sites for RBR (Nowack et al. 2012; Dissmeyer et al. 2009), and after releasing from RBR, E2F activates CDKB1;1 expression (Boudolf et al. 2004). Complete inhibition of CDKB activity is required for endoreplication, and this is not possible unless CDKA;1 activity is inhibited; therefore, our results provide a model in which *SIM* is inhibiting both types of CDKs to prevent the G2/M transition phase, to establish endoreplication in the developing trichomes.

SIM functions can be partially predicted on the basis of their conserved sequence similarity. Alignment of *Arabidopsis* SIM and most of the SMRs proteins show similarity through five conserved motifs (Churchman et al. 2006). Bio-informatics work discussed in chapter-3 of my dissertation, have found more than 400 *SMRs* gene family members and all at least shared motif-1 and motif-2.

SIM motif-1 (SIM C33---P44, CT**TP**TSSDHKIP) is most conserved and T-35 followed by proline is a potential phosphorylation target site. Making the site directed mutants of the SIM protein targeting the most conserved amino acid residues provides clues about their functionality. The mutation of T34T35P36 to AAA fails to complement *sim* phenotype. SIM has a total of

three threonine amino residues, which are followed by proline, meeting the basic requirement to be potential phosphorylation target site of the CYC/CDKs complexes. Substitution of threonine-35 (T35) to alanine (A35) fails to complement *sim* phenotype of *Arabidopsis* trichomes (Chapter-4), whereas phosphomimic mutation to aspartic acid (T35D), partially complements *sim* phenotype in T2 generation and completely complements in homozygous lines. Therefore, threonine-35 phosphorylation seems necessary for the SIM protein function. SIM-T50AT62A complements *sim* phenotype, while T35AT50AT62A fails to complement *sim* phenotype, which further supports the notion that T35 is a phosphorylation target site. Therefore, all available evidence in chapter-4 suggests that T35 phosphorylation seems necessary for SIM function.

The sequence in motif-3 (SCKRKL) and motif-5 (KRRS) seems to be "nuclear localization sequences" (Kosugi et al. 2009). After deletion of Motif-5 and 4, SIM was expressed in both nucleus and cytoplasm of tobacco leaf. Deletion of motif-5, motif-4 and motif-3 shifted expression of SIM from nucleus almost to the cytoplasm. These results showed that the true NLS sequence is likely to be in motif 3 and motif 5, while motif 4 seems to play a small role. Motif-4, a cyclin binding site in the animal Cip/Kip kinase inhibitors, does not seem necessary for the SIM function. Over-expression of Enhanced Yellow Fluorescence Protein (eYFP) tagged SIM-motif-4, in which the six amino acid residues were mutated, does not change localization of SIM in *Arabidopsis* trichome and complement *sim* phenotype. We further narrowed down our focus to the conserved amino acids, notating with underlined amino acid sequences in Motif 3 (SC<u>KRK</u>L), Motif 5 (<u>KRRS</u>), or both Motifs 3 and 5 to Alanine (A). Mutation of either motif-3 or motif-5 leads to SIM expression in both nucleus and cytoplasm, whereas mutation of both motif-3 and 5 completely changed the localization of SIM from nucleus to cytoplasm and failed to complement *sim* phenotype as well. On the basis of the available results in chapter-4, SIM

nuclear localization sequences are located in motif-3 and 5. Considering SIM phosphorylation data and SIM ability to inhibit CDKs, we can propose that SIM and CDKA;1 work in a feedback loop where SIM is phosphorylated by CDKA;1 and phosphorylated SIM inhibits CDKA;1 activity. Hence, SIM controls both CDKA;1 and CDKBs activity, and CDKA;1 regulates both SIM and CDKBs activity (Figure 6.1).

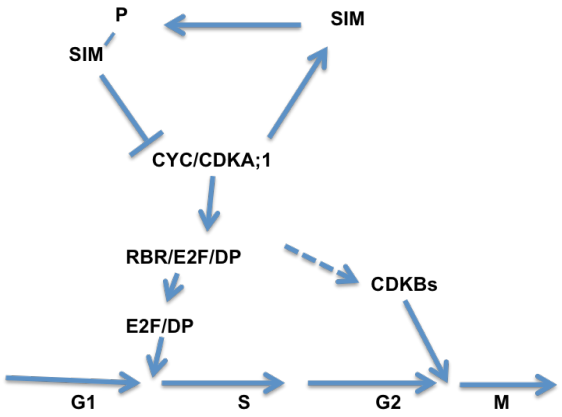


Figure 6.1 Proposed model of SIM functions in the cell cycle

Stability is a crucial determinant of a protein function. Substitution of both lysine residues of SIM in motif-3 (SCKRKL) and its expression under GL2pro gives similar phenotype as overexpression of another class of plant CDK inhibitor Kip-related protein (KRP). So, we assumed that these amino acids might responsible for the protein degradation via ubiquitination-mediated pathway because mutation in these amino acid make SIM more stable. However, stability assay in this study (chapter-5) have shown that SIM is degraded by 26 proteosome, and SIM- K73A K75A stability is not affected with MG132, a proteosome inhibitor. More investigation is needed to find the exact amino acid sequence determinant for SIM protein degradation.

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VITA

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